



Separation and characterisation of biomolecules in effluent from the herring industry

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Separation and characterisation of biomolecules in effluent from the herring industry



PhD Thesis
Nina Gringer
2015

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PhD thesis
Nina Gringer

Division of Industrial Food Research
National Food Institute
Technical University of Denmark

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DATA SHEET

Title: Separation and characterisation of biomolecules in effluents from the herring industry

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Front page: Picture of fillet ripened spice-cured herring (right) and traditional barrel-salted spice-cured herring (left)

PREFACE

This PhD project was carried out at the National Food Institute, Technical University of Denmark, under the supervision of Caroline P. Baron and Henrik H. Nielsen. The project was funded by Nordic Innovation and the Technical University of Denmark.

The aim of the present project has been to characterise the brines generated during the final ripening of marinated herring (*Clupea harengus*) products, and to test two separation technologies on these brines with the main focus on recovery of bioactive compounds. This PhD project is part of a larger project called PIPE – *Pelagic industry processing effluents, innovative and sustainable solutions*, in which we considered all the process waters from the marinating herring industry, from boat to glass jar. Thus several industry partners have contributed with knowhow and with valuable discussions for which I am truly grateful. A special thanks to Tore Svenson from LiqTech A/S and Jørgen Andersen from Lykkeberg A/S.

The overall design of the experimental work have been discussed, carried through and evaluated in close collaboration with Ingrid Undeland from Chalmers University of Sweden, who I also wish to thank.

I also would like to thank my supervisors, Caroline P. Baron and Henrik H. Nielsen for making this PhD possible for me, and especially Caroline P. Baron for her guidance, encouragement and for believing in me.

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Last, but not least, I would like to thank my family for all their help and support – especially my girls, Olivia and Nikoline, and my always patient husband Thomas.

Nina Gringer

SUMMARY

In the production of marinated herring, a traditional Scandinavian product, up to 7 m³ of wastewaters are generated per tons processed herring, of which no attempt is made to collect and utilise the organic matter. Today, the majority of fishery by-products are used to produce low profitable products like e.g. fish oil and fish meal. Better profitability is obtained by producing human consumables from bioactive compounds, such as peptides, amino acids and phenolics. Such bioactive compounds can be used in food products as antioxidants to prevent or delay the oxidation of fats and oils. As a consequence, separation and purification methods that results in isolation of molecules with antioxidant activity from the original natural source, e.g. waste or wastewater, is needed.

This PhD project aimed at characterising the herring brines generated during the final ripening stage in the production of marinated herring, and at identifying bioactive compounds in the brines. Furthermore, two separation techniques were tested and analysed for their applicability in the recovery of the bioactive compounds; electro-flocculation (EF) and ultrafiltration (UF) using a silicon carbide (SiC) membrane.

The thesis encompassed proximate investigation of all types of final ripening brines; from traditional barrel-salted herring with (TSp) and without (TSa) spices and the corresponding desalting brines, D-TSp and D-TSa, respectively, and from fillet ripened spice-cured (SC) and vinegar-cured (VC) herring. All brines were loaded with protein, especially SC, TSa and TSp which had protein concentrations of 41.7, 48.4 and 56.7 mg/mL, respectively. The brines were further tested for antioxidant and enzymatic activity, and whereas all six brines showed high antioxidant and protease activity, only TSa and TSp had peroxidase activity. The two spice-containing brines, TSp and SC, were used to test the two separation technologies, yet due to high aluminium concentrations (<1.7 g/L) in the EF-treated brine, it was concluded that this technology is not applicable as a recovery technology. The combination of pre-filtration, with a 50 µm polypropylene filter, and ceramic UF resulted in retentions of up to 42% COD (chemical oxygen demand), more than 95% TSS (total suspended solids), more than 85% iron, up to 44% nitrogen, 100% fatty acids and more than 80% protein. Additionally, the phenolic content was measured in TSp and SC, in which the level were found to be 3.7 mg GAE/mL and 2.6 mg GAE/mL (GAE, gallic acid equivalents). Thus these two brines were selected for further investigation of the low molecular weight (LMW) bioactive compounds, such as peptides, amino acids and phenolics as well as the antioxidant activity. Moreover, to evaluate if the antioxidant activity is caused by the peptides or the phenolics, TSa were included in this study as well. The three brines were dialysed, and the <10 kDa fraction were further fractionated using size exclusion chromatography (SEC). 94 SEC-fractions were generated and the protein-enriched fractions were pooled into P1, P2 and P3. Amino acids, phenolic acids and peptide sequences were detected in the three pooled samples. Results

showed that the radical scavenging activity was mainly following the phenolics, whereas iron chelating activity and reducing power were to a higher extent following the peptides. Furthermore, the results showed that the processing method, rather than the addition of spices to the ripening brine, was responsible for the LMW composition and antioxidant activity. To further test the usage of these three brines their ability to act as a source of natural antioxidants were tested; as coating agents for frozen herring and as additive in fresh herring mince. The test revealed that TSa and TSp were potent coating agents whereas SC was better in preventing oxidation as an additive in herring mince. Besides the final ripening brines, the process waters generated during the first steps in the production of marinated herring were correspondingly analysed for their proximate composition. In total, it was found that per ton of processed herring up to 119 kg protein and 44 kg fatty acids are discarded as wastewater.

In conclusion, this work shows that huge amounts of protein and fatty acids, as well as bioactive peptides, amino acids and phenolic compounds are presently discarded as waste in the marinated herring industry. Although some optimisation is still needed, the ceramic UF treatment showed its potential as a separation technique of these brines. With proper recovery treatment, the starting point for creating value-added products from herring brines and by that increasing the revenue for the herring producers has been defined by this work.

RESUMÉ

Under fremstilling af marinerede sild, et traditionelt Skandinavisk produkt, genereres der op til 7 m³ spildevand per ton forarbejdet sild, og der gøres intet forsøg på at indsamle og udnytte det organiske materiale. I dag bliver størstedelen af biprodukterne fra fiskeindustrien anvendt til fremstilling af produkter som f.eks. fiskeolie og fiskemel med lavt økonomisk udbytte. En højere profit kan opnås ved fremstilling af humane konsumvarer fra bioaktive forbindelser, såsom peptider, aminosyrer og fenoler. Sådanne bioaktive forbindelser kan anvendes i fødevarerprodukter som antioxidanter for at forhindre eller forsinke oxidationen af fedtstoffer og olier. Som en konsekvens heraf er der et behov for separations- og oprensningsmetoder, der resulterer i en isolering af molekyler med antioxidant aktivitet fra den oprindelige naturlige kilde, f.eks. fra affald eller spildevand.

Dette Ph.d.-projekt har haft til formål at karakterisere de sildelager, der genereres under den sidste modningsfase i produktionen af marinerede sild, samt at identificere bioaktive forbindelser i lagerne. Desuden blev to separeringsteknikker testet og analyseret for deres anvendelighed i oprensningen af bioaktive forbindelser; elektro-flokkulering (EF) og ultrafiltrering (UF) ved brug af siliciumkarbid (SiC) membraner.

Afhandlingen omfattede en karakterisering af alle typer af modningslager; fra gammeldagsmodnet saltsild både med (TSp) og uden (TSa) krydderier og de tilsvarende afsaltningslager, henholdsvis D-TSp og D-TSa, og fra filetmodnet kryddersild (SC) og syremodnet sild (VC). Alle lagerne indeholdt høje koncentrationer af protein, især SC, TSa og TSp som havde proteinkoncentrationer på hhv. 41,7, 48,4 og 56,7 mg/mL. Sildelagerne blev yderligere testet for antioxidant og enzymatisk aktivitet, og mens alle seks lager udviste høj antioxidant og protease aktivitet, var det kun TSa og TSp, der havde peroxidase aktivitet. De to krydderiholdige lager, TSp og SC, blev brugt til at teste de to separationsteknologier, men på grund af høje aluminiumkoncentrationer (<1,7 g/L) i de EF-behandlede lager, blev det konkluderet, at denne teknologi ikke er velegnet til oprensning. Kombinationen af for-filtrering, med et 50 µm polypropylen filter, og keramisk UF resulterede i retentioner på op til 42% COD (kemisk iltforbrug), mere end 95% TSS (total suspenderede stof), mere end 85% jern, op til 44% nitrogen, 100% fedtsyrer og mere end 80% protein. Derudover blev indholdet af fenoler målt i TSp og SC, hvor niveauet blev fundet til at være henholdsvis 3,7 og 2,6 mg GAE/mL (GAE, gallussyre ækvivalenter). På baggrund af dette blev disse to lager udvalgt til yderligere analyser af de bioaktive forbindelser med lav molekylvægt (LMW), såsom peptider, aminosyrer og fenoler samt antioxidant aktivitet. For at kunne vurdere om den antioxidant aktivitet er forårsaget af peptider eller fenoler blev TSa også inkluderet i dette studie. De tre lager blev dialyseret og <10 kDa fraktionen blev yderligere fraktioneret ved hjælp af størrelseskromatografi (SEC). 94 SEC-fraktioner blev genereret og de proteinberigede fraktioner blev samlet til P1, P2 og P3. Aminosyrer, fenolsyrer og peptidsekvenser blev

detekteret i de tre samlede prøver. Resultaterne viste, at radikal scavenging aktivitet hovedsageligt fulgte fenolerne, mens jern chelation aktivitet og reducerende effekt i højere grad fulgte peptiderne. Endvidere viste resultaterne, at modningsprocessen, snarere end tilsætning af krydderier til sildelagen, var ansvarlig for sammensætningen i LMW og den antioxidative aktivitet. For yderligere at teste brugbarheden af disse tre lager blev deres evne til at fungere som en naturlig kilde af antioxidanter testet; som coating på frosne sild og som tilsætningsstof i friskhakket sildekød. Testen viste, at TSa og TSp var potente coatingmidler, mens SC var bedre til at forebygge oxidation som tilsætningsstof i det friskhakkede sildekød. Udover modningslagerne blev det genererede procesvand fra de første trin i produktionen af marinerede sild ligeledes analyseret for deres overordnede sammensætning. I alt blev det fundet at der, per ton produceret sild, kasseres op i mod 119 kg protein og 44 kg fedtsyrer med spildevandet.

Det kan konkluderes, at store mængder af protein og fedtsyrer, samt bioaktive peptider, aminosyrer og fenolforbindelser i øjeblikket kasseres som affald i den marinerede sildeindustri. Selvom der stadig er behov for optimering er potentialet for keramisk UF som separationsteknik af sildelagerne vist. Med den rette oprensningsteknik, er udgangspunktet for at skabe værdiforøgende produkter ud fra sildelagerne, og dermed øge indtægterne for sildeproducenterne, blevet defineret via dette arbejde.

ABBREVIATIONS

A*	Antioxidant radical
AH	Antioxidant molecule
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BOD	Biological oxygen demand
BOD ₅	BOD demand in 5 days at 20°C
CA	Cellulose acetate
C _{feed}	Concentration in feed
COD	Chemical oxygen demand
CP	Concentration polarisation
C _{permeate}	Concentration in permeate
DAF	Dissolved air flotation
DHA	Docosaheptaenoic acid
D-TSa	Desalting brine TSa
D-TSp	Desalting brine TSp
EF	Electroflocculation
EPA	Eicosapentaenoic acid
FOG	Fat, oil and grease
FPCP	Fish protein co-product
FPH	Fish protein hydrolysate
HMW	High molecular weight
ISP	Isolated soy protein
L	Lipid
LC-PUFA	Long chain polyunsaturated fatty acid
LMW	Low molecular weight
MF	Microfiltration
MSP	Membrane separation process
MWCO	Molecular weight cut-off
NF	Nanofiltration
PAN	Polyacrylonitrile
PES	Polyethersulfone
PP	Polypropylene
PSO	Polysulfone
PVDF	Polyvinylidene difluoride
PVP	Polyvinyl-pyrrolidone
PW	Processing water
R	Rejection
R*	Unstable radical
RO	Reverse osmosis
ROO*	Peroxy radical
ROOH	Hydroperoxide
ROS	Reactive oxygen species

RSW	Refrigerated sea water
SB	Salt brine
SC	Brine from spice-cured herring
SiC	Silicon carbide
SW	Storage water
TMP	Transmembrane pressure
TPC	Total phenolic content
TSa	Brine from traditional barrel-salted herring
TSp	Brine from traditional barrel-salted spice-cured herring
TSS	Total suspended solids
UF	Ultrafiltration
VC	Brine from vinegar-cured herring
W	Water
WPC	Whey protein concentrates

Amino acids

A	Alanine, Ala
C	Cysteine, Cys
D	Aspartic acid, Asp
E	Glutamic acid, Glu
F	Phenylalanine, Phe
G	Glycine, Gly
H	Histidine, His
I	Isoleucine, Ile
K	Lysine, Lys,
L	Leucine, Leu
M	Methionine, Met
N	Asparagine, Asn
P	Proline, Pro
Q	Glutamine, Gln
R	Arginine, Arg
S	Serine, Ser
T	Threonine, Thr
V	Valine, Val
W	Tryptophan, Trp
Y	Tyrosine, Tyr
	Hydroxyproline, Hyp

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1. Introduction and objectives

Today, the majority of fishery by-products are used to produce fish oil, fish meal, fertiliser, pet food and fish silage (Penven et al., 2013), and most of these products possess low economic value. In general, a far better profitability is obtained by producing human consumables and currently the highest profitability is expected from bioactive compounds (Kim and Mendis, 2006). Bioactive peptides are usually 2-20 amino acid residues in length, and depending on the amino acid sequence they may exhibit a range of activities such as immunomodulatory, antimicrobial, antihypertensive and antioxidant actions, and some are even multifunctional (Hartmann and Meisel, 2007; Kim and Mendis, 2006). Some bioactive compounds are phenolics. These are widely distributed in nature and are the most abundant antioxidants in the diet e.g. in components of fruits, spices, herbs, vegetables, and beverages. These antioxidants are used in food products to prevent or delay the oxidation of fats and oils (Decker et al., 2005), and in order to replace some of the synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), there is an increasing focus on recovery of natural food antioxidants (Ito et al., 1986). The interest in the use of natural antioxidants, peptides or phenolics, in the food industry, not only for application as preservatives but also because of their benefits to human health, has been growing (Shahidi and Zhong, 2010; Barbosa-Pereira et al., 2013), especially in compounds from marine origin such as algae, fish/shellfish and marine bacteria (Shahidi and Amarowicz, 1996; Amarowicz and Karamać, 1999; Athukorala et al., 2003). Thus, separation and purification methods, that results in isolation of molecules with antioxidant activity from the original natural source (e.g. waste or wastewater), is needed.

In the production of marinated herring, a traditional Scandinavian product, huge amounts of wastewaters are generated. There are several types, for instance seawater during transport, filleting water and water for making salt brine, and each type have a specific composition and degree of organic matter and the different types are generated in very diverse quantities. All the water (up to 7 m³/tons herring) is usually pooled at the “end of the pipe” and discarded as wastewater without any attempt to collect and utilise the organic matter. As the process waters are considered as food grade, it is of uttermost importance that the recovery of these molecules is conducted without introducing chemicals or altering the molecules of interest. Among the technologies available for treatment and separation of wastewater many do introduce chemicals to the treated water or alter the molecules during the process and thus cannot be used in the recovery of these molecules. Moreover, some treatment technologies are inconvenient for small local herring producers, as they would require skilled technical support for proper handling, which would be too expensive for a small or medium sized industry (El-Mashad and Zhang, 2007).

This PhD thesis is part of a large project called PIPE; *Pelagic industry processing effluents innovative and sustainable solutions*. The aim of the PIPE project was to characterise all the effluents from the marinating herring industry, from boat to glass jar, and identify bioactive compounds in the different process waters and ideally propose possible use of these biomolecules. Furthermore, two separation techniques was pre-selected to be tested and analysed for their usability in the recovery of bioactive compounds from the marinating herring industry; electro-flocculation and ultrafiltration using a silicon carbide (SiC) membrane. My part in this project was to focus on the process waters from barrel to glass jar; the characterisation, separation and subsequent evaluation, and the detection of bioactive compounds in these effluents. The working hypotheses throughout my work have been;

As herring are known to be healthy, the brine generated throughout long periods of salting and marinating contain bioactive compounds from the herring, and these compounds can be recovered (by electro-flocculation and ceramic ultrafiltration) and used as an added value, e.g. in functional foods or nutraceuticals, instead of being discarded as waste.

The working hypothesis is further divided in three main objectives;

- 1) *Characterise the wastewaters generated from barrel to glass jar*
- 2) *Test and evaluate the selected separations methods on the wastewaters*
- 3) *Recover bioactive compounds from the wastewaters*

The background for these objectives are provided through reviewing and discussing the production of marinated herring and the corresponding brine (**Chapter 2**), the available separation and treatment technologies for food industry wastewaters (**Chapter 3**) and the bioactive compounds from fish processing by-products (**Chapter 4**). The focus in these three chapters is based on the known knowledge prior to this PhD work, and thus is not including the discussion of my work. Five papers have been completed from the PIPE-project (**Chapter 5**). Paper I, II and III are presenting the experimental work directly related to my PhD; the characterisation of the wastewaters generated from barrel to glass jar, the testing of the selected separation methods, and the recovery of bioactive molecules from these waters. The two supporting papers, Paper A and B, are dealing with the process waters from boat to barrel and testing of the selected separation methods on these waters (Paper A), and evaluating the usability of the wastewaters from barrel to glass jar as coating agents and additives in preventing oxidation (Paper B).

The experimental work is schematically presented in **Figure 1.a** and elaborated on in the discussion in **Chapter 6**. **Chapter 7** is answering the working hypotheses of this work, and providing the conclusions and perspectives.

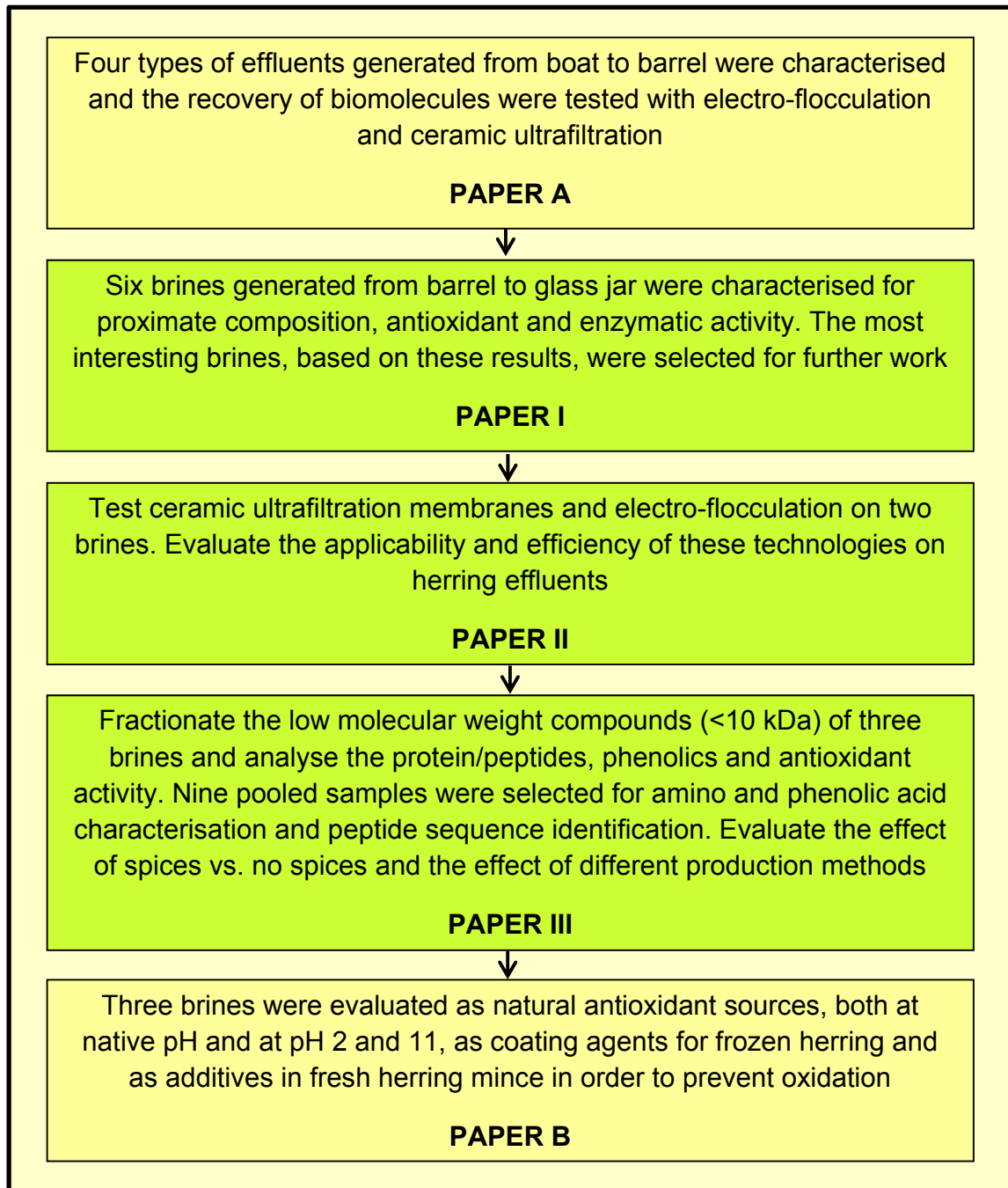


Figure 1.a. Schematic overview of the experimental work presented in paper A, I-III and B.

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2. Herring production

Atlantic herring (*Clupea harengus*) is a small fatty pelagic fish (20 to 40 cm), widely distributed on both sides of the Atlantic Ocean (FAO, 1985). Out of the top 10 species contributing to the world catches, five of them are small pelagic fish species and the Atlantic herring (from now on referred to as herring) was one of these, which in 2004 yielded the fifth highest catch of all species (FAO, 2007). It is rich, for example, in n-3 long chain polyunsaturated fatty acids (LC-PUFAs), vitamin D and high value protein, and its consumption is linked with improved health benefits (Lindqvist et al., 2007; 2009). Herring form large schools particularly during feeding and spawning periods. In 2009, 256,000 tons of herring were landed in the Northwest Atlantic (FAO Area 21) and 2,254,000 tons were landed in the Northeast Atlantic (FAO Area 27) of which most were fished in the Russian Federation, Norway, Iceland and the Faroe Islands (FAO, 2011).

In Northern Europe there is a long tradition of producing and consuming herring as a marinated product. In Denmark herring is one of the most important species in the fishing industry. In 2012 approx. 125,000 tons of herring were landed in Denmark, which were 45% more than in 2011 (Statistics Denmark, 2013). Herring has always been of special interest in the Scandinavian countries and the production of salted herring (a traditional preservation process) has been carried out for centuries (Cutting, 1955), probably originating in the eight century (Voskresensky, 1965). The process has during the years been changed and improved by the various countries that produce salted herring. It has been automated to some extent and standardised recipes has been developed that give salted herring with the wanted salt content and taste. Today, the preservation of the herring is not the main purpose; instead the process is now carried out to obtain a well-ripened product with a tender consistency and a pleasant taste and odour (Stefánsson et al., 1995).

The salting process results in considerable changes in the herring muscle during cold storage, and thus can be considered to consist of two stages; salting and ripening. The salting stage includes an initial soaking of the fish in salt, which initiates the extraction of fluids from the fish and creates a natural brine (Vokresensky, 1965). Hereafter, a saturated brine consisting of water and salt is added. When the salt concentration in the tissues equals that of the surrounding brine the ripening stage takes over. The ripening consists of both chemical and biochemical processes that change the characteristics of the fish tissues and thus the sensory properties of the final fish product. Ripening is believed to be caused by enzymes, as the enzymes will breakdown macromolecules in the herring musculature, e.g. peptides, amino acids and fatty acids, which will eventually change the texture of the fish (Nielsen, 1995). The result of the ripening is consequently a soft and tender product with the pleasant taste associated with salted herring. During salting of fish, it has been found that the product yield

compared to raw material weight is increasing (Birkeland et al., 2005). Hence, the protein undergoes a degradation process and, as a consequence, the protein fractions will be extracted into the brine and the concentration of protein in the brine will increase during the marinating process (Andersen et al., 2007; Stefánsson et al., 1995). The degradation products found in the brine are soluble nitrogenous compounds like peptides, free amino acids and myofibrillar protein (Nielsen, 1995). Svensson and Andersen (2014) reported an increase in the protein content in the brine to approx. 3 g/100 g after 60 days of storage, and from day 60 to day 277, an increase from 3 g/100 g to 5 g/100 g was observed. Moreover, they reported that the protein content still seemed to increase until the last day of sampling (day 277). Andersen et al. (2007) reported that the protein content in the brine of salted herring increased from around 1% at day 2 to 8% at day 371, with an initial fast increase up to day 100. Simultaneously, the protein content in the herring muscles decreased slowly from 18% to 14% in the same period, which means potentially protein and bioactive compounds, such as peptides, to the brine are leaching from the fish to the brine. If acetic acid is also added to the brine the pH will decrease, and this causes protein denaturation and lower water absorption (Kolakowski and Bednarczyk, 2002), and hence a lower product yield.

During the normal biological cycle of herring, the content of fat and other components varies (Oterhals, 1995; Aro et al., 2000). The food industry takes advantage of this in the production of marinated, canned, frozen, smoked, and salted fish as the fat content differs in various seasons. The best products are obtained from herring salted during the feeding stage, which normally lasts for only a few months, when the endogenous proteolytic activity is high as well as the fat content. Nielsen et al. (2005a) reported the lipid contents in different catches during the seasons to be in the range from 1.3 to 25.7% and the variation within catches was found to be correspondingly high. They found a strong linear relationship between the lipid (L) and the water (W) content ($L = -1.014 \times W + 82.274$, $r^2 = 0.928$, $P < 0.001$). It is generally recognised that the sum of water and lipids in the herring muscle is constant at 80%, as the amount of protein, minerals and fibres is constant (Vogt et al., 2000; 2002). However, the results reported by Nielsen et al. (2005a) showed that the sum increased as the lipid content increased. The water and lipid content have been reported to vary between 640 and 810 g/kg and 20 and 170 g/kg, respectively, and the herring caught in the North Sea in September were reported to have the highest lipid content, while the lipid content was lowest in herring caught in the North Sea during February (Nielsen et al., 2005b).

2.1. Herring brine products

The marinated herring products, investigated in this PhD, are processed according to old family traditional recipes from herring caught in the North Atlantic. Four different basic products are produced, which are turned into several different end-products depending on the final brine added to the product, see **Figure 2.a**. The four basic products are;

- Traditional barrel-salted herring (TSa)
- Traditional barrel-salted spice-cured herring (TSp)
- Spice-cured herring (SC)
- Vinegar-cured herring (VC)

The herring is landed in Norway, Sweden, Iceland or Germany where it is pre-processed at local herring producers prior to arrival to the end-producer in Denmark. The two traditional barrel-salted products (TSa and TSp) are produced by mixing 100 kg of whole-headed herring with 10 kg of salt in a 100 L plastic barrel. After 24 h, the barrels are filled with saturated salt brine and stored at 0-5°C for ≥6 month. The herring is then filleted and the fillets placed back in the brine, which was created during the first half year, for additionally 6-18 month during which the barrels are transferred to the end-producer in Denmark. On costumers demand the barrels are emptied and approx. 35 L heavy loaded brine is discarded per 100 kg herring. The herring is then desalted in fresh water for 16-20 h, which creates approx. 70 L desalting brine (D-TSa and D-TSp) per 100 kg herring. The desalted herring fillets are packed with freshly prepared brine containing the desired combination of salt, sugar, spices and preservatives, closed and distributed to the consumers (Lykkeberg, 2014).

The spice-cured (SC) and vinegar-cured (VC) herring are produced in a much faster manner, and consequently end up as cheaper products with a different characteristic texture compared to the traditional barrel-salted products. The herring are still caught and landed in Norway, Sweden, Iceland or Germany and pre-processed here before transport to the end-producer in Denmark. Upon landing, herring are headed, gutted, de-skinned and filleted and some are cut into bites. The fillets/bites are covered with brine containing either salt, sugar and spices (SC) or salt and acidic acid (VC) and stored for a minimum of 42 and 35 days, respectively, however, often for a longer period. During packaging of the final SC product, approx. 40 L of heavy loaded spice brine is discarded and the fillets/bites are covered with fresh spice brine and distributed to the costumers. Before the VC fillets/bites are ready for packaging the herring is covered with a sugar brine for 1-14 days to extract the heavy vinegar taste and to sweeten the product. Approx. 80 L of vinegar brine and 100 L of sugar brine are discarded per 100 kg herring (Lykkeberg, 2014).

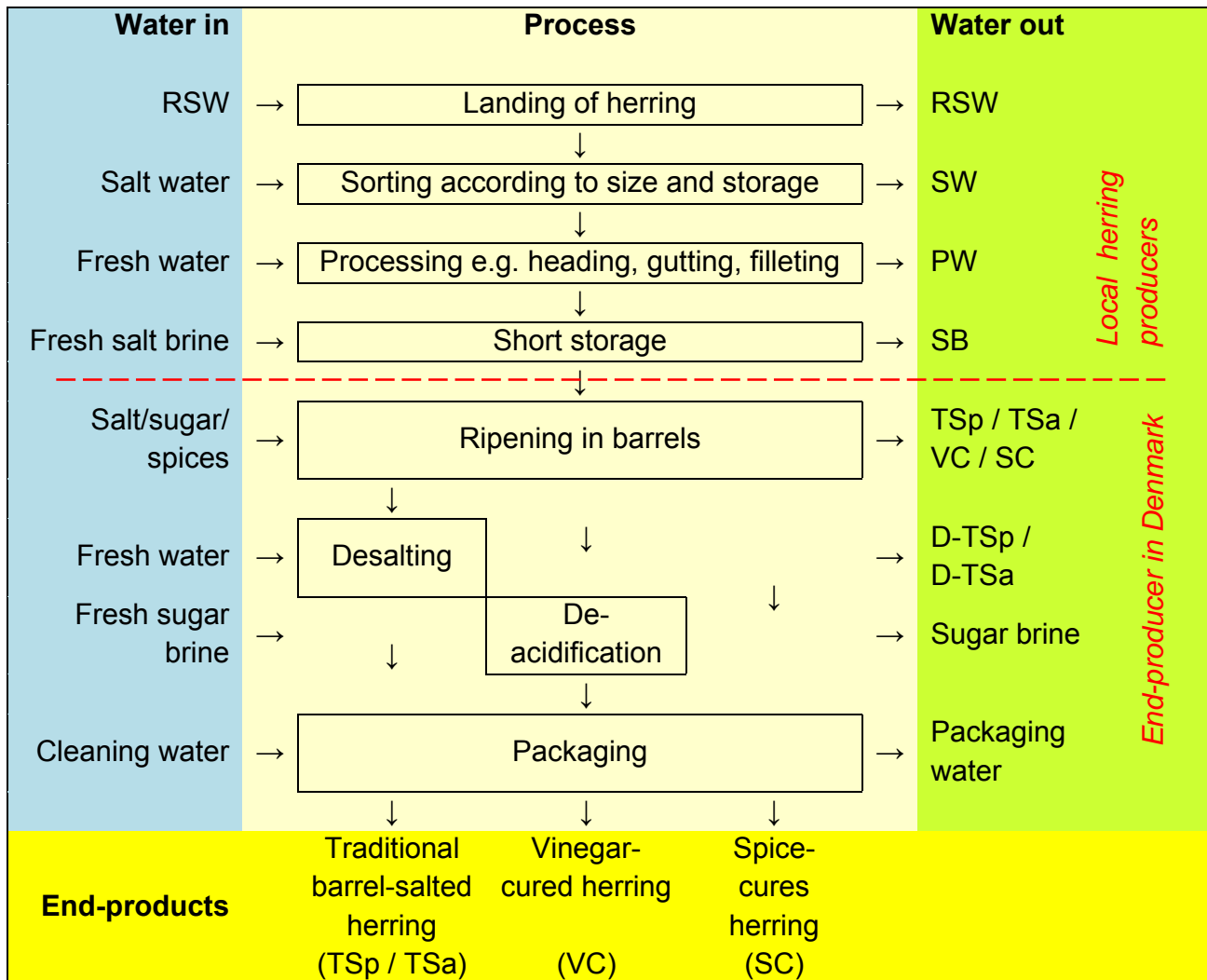


Figure 2.a. Flow chart for the production of marinated herring. Water in and out is indicated as well as steps taken place at the local herring producers, where the herring is landed, and at the end-producer in Denmark. RSW, refrigerated sea water; SW, storage water; PW, processing water; SB, salt brine; TSp, brine from traditional barrel-salted spice-cured herring; TSa, brine from traditional barrel-salted herring; VC, brine from vinegar-cured herring; SC, brine from spice-cured herring; D-TSp, desalting brine from traditional barrel-salted spice-cured herring; D-TSa, desalting brine from traditional barrel-salted herring.

Besides the very loaded brines, generated during the last part of the production, the process of producing marinated herring creates several types of wastewater streams (see **Figure 2.a**); 1) refrigerated sea water (RSW) in which the herring is stored during transport on the sea to the processing site, 2) storage water (SW), which is imitated sea water in which the herring is

kept after sorting according to size and until processing, 3) processing water (PW) which is the water used during processing into different cuts, i.e. a headed and gutted product, de-skinned fillets, and bites of de-skinned fillets, and finally 4) a salt brine (SB) in which the different herring cuts are placed for a short time period until packaging in the barrels (Paul Mattsson, 2014). These waters are described and analysed more in detail in Paper A.

The amount of wastewater generated during the entire process is enormous, and since the European Union, together with Norway, Iceland and Liechtenstein, annually lands more than 1 million tons of herring for human consumption, of which most ends up as marinated product (Eurostat, 2014), there is a huge amount of liquid by-products available for value adding from this production alone in Europe. However, knowing that the process waters are available in huge amount are not enough; sufficient treatment will be needed to recover the value without altering or destroying the desirable compounds. In the next chapter, **Chapter 3**, the available treatment technologies for food industry liquid waste are reviewed.

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3. Treatment of food industry liquid waste

Treatment of wastewater is getting more and more important, as the world population is increasing and the standard of living is as well. One of the consequences is an increasing production of solid, liquid and gaseous wastes which in turn give rise to environmental problems. The costs for disposal of wastewater and the environmental regulations are two major driving forces in the use of treatment technologies for recovery of valuable products from waste streams and for effectively recycling of water. The major purpose of using the various available treatment technologies is to reduce environmental pollution by waste or wastewater discharge, and thus the recovery of valuable products for human, animal or industrial uses is, in many cases, only a secondary purpose (El-Mashad and Zhang, 2007).

Large quantities of both liquid and solid wastes are produced annually by the food processing industry. These waste materials contain principally biodegradable organic matter and disposal of them creates a significant potential to pollute both land, air, and water because of its high chemical oxygen demand (COD) (Hang, 2004). The COD is a measure of the amount of oxygen needed to chemically oxidise the wastes in a specific time and temperature, and the level can be as high as 90,000 mg/L or more, which is more than 100 times greater than common domestic sewage. The waste may also contain a moderately high salt or acidity level and might be contaminated with pathogens, but only rarely the food processing waste contains significant amounts of toxic chemicals (Hansen and Cheong, 2007). Thus, for many industries there is a need to treat the waste and wastewater in order to cope with specific guidelines and more stringent standards (provincial or local government). In the fish processing industry the wastewater generated should be treated through a good waste management and treatment technology prior to discharge (Chowdhury et al., 2010). The aim is to obtain a pollutant-free effluent, which could be either disposed of or recycled to the process, and a sludge, which could be turned into a value-added compound/product (Coca et al., 2011).

This chapter describes the most commonly used treatment technologies for wastewater generated in the food industry. Generally, a lot of process steps are executed during food production, and treatment technologies are often conducted on all wastewaters collected at the “end of the pipe”. However, if the purpose of the treatment is also to recover valuable biomolecules, the applied technologies could be conducted on the wastewater generated from a single unit operation. In the PIPE project, the aim was to recover potential high value biomolecules, and as the different process waters were believed to have significantly different profiles, the tested methods were conducted on each collected type of water. Furthermore, some of the wastewaters generated are food-grade (i.e. the brine generated during the ripening period), while others cannot be considered directly for consumption (e.g. the RSW).

Food-grade quality refers to the minimum standard for substances to qualify as fit for human consumption, (FDA, 2014), and thus different treatment steps will be necessary to recover the biomolecules from the waters, and prepare them for use in food or nutraceuticals.

3.1. Treatment types for food processing wastewater

The treatment of wastewater is usually described in means of primary, secondary and tertiary treatment (Coca et al., 2011). As shown in **Figure 3.a**, a pre-treatment step is often included, before the primary treatment, in which the coarse particles are removed with a grid or a screening step. The *primary treatment* consists of physical separation steps, e.g. sedimentation, by which the suspended solids are allowed to settle. For oily wastewaters this first step is used to remove free oils from the emulsion or suspension and it is common to use techniques such as gravity and centrifugal separations. The *secondary treatment* is intended to reduce the organic load that remains after the primary treatment, by combining a biological treatment (anaerobic or aerobic) with a following secondary sedimentation. In oily wastewater, the aim is to break oil-in-water emulsions and to remove the dispersed oil, and chemical treatment, flotation, and membrane filtration (microfiltration, MF, and ultrafiltration, UF) are common techniques for this step. The *tertiary treatment* is a physicochemical process that reduces the levels of dissolved organic and inorganic compounds. The main treatments used are membranes (MF, UF, or reverse osmosis, RO), activated carbon adsorption and evaporation. For oily wastewater these processes are used to remove finely dispersed, emulsified and soluble oil fractions (Coca et al., 2011; Bolzonella et al., 2007; Klemeš and Perry, 2007).

In the present work a sieve was used as a pre-treatment step to remove pieces of herring and scales left in the wastewaters before testing the selected technologies. At the local herring producers a primary treatment is often conducted, in which the majority of the fat is removed prior to leading the treated wastewater to the community treatment plant. One of the selected technologies, electro-flocculation, is also a primary treatment technology and the other, membrane filtration, is categorised as a secondary treatment. These methods are all elaborated on in **Section 3.2**.

Although this work did not test any chemical or biological treatment methods, it should be noted that anaerobic treatment is a very used and well suited technique for fish processing wastewater, as a high degree of BOD₅ (biological oxygen demand by microorganisms during the first 5 days of biodegradation at 20°C (Coca et al., 2011)) removal can be achieved at a significantly lower cost than in comparable aerobic systems. In addition, smaller quantities of highly stabilised, and more easily dewatered, sludge will be generated and the methane-rich gas, which is produced, can be captured for use as a fuel (Chowdhury et al., 2010; Johns,

1995). Fish processing industries require a large amount of salt (NaCl) for fish conservation and the wastewater generated is thus rich in salts together with protein-based nitrogen and organic matter. The presence of high sodium or chloride concentrations will in general inhibit the anaerobic treatment of wastewater as well as methanogenesis (formation of methane by microbes) (Lefebvre and Moletta, 2006). However, the treatment of saline wastewaters (1.5 to 15% w/v NaCl) in several industries has been demonstrated using anaerobic treatment, at least at the pilot scale (Xiao and Roberts, 2010).

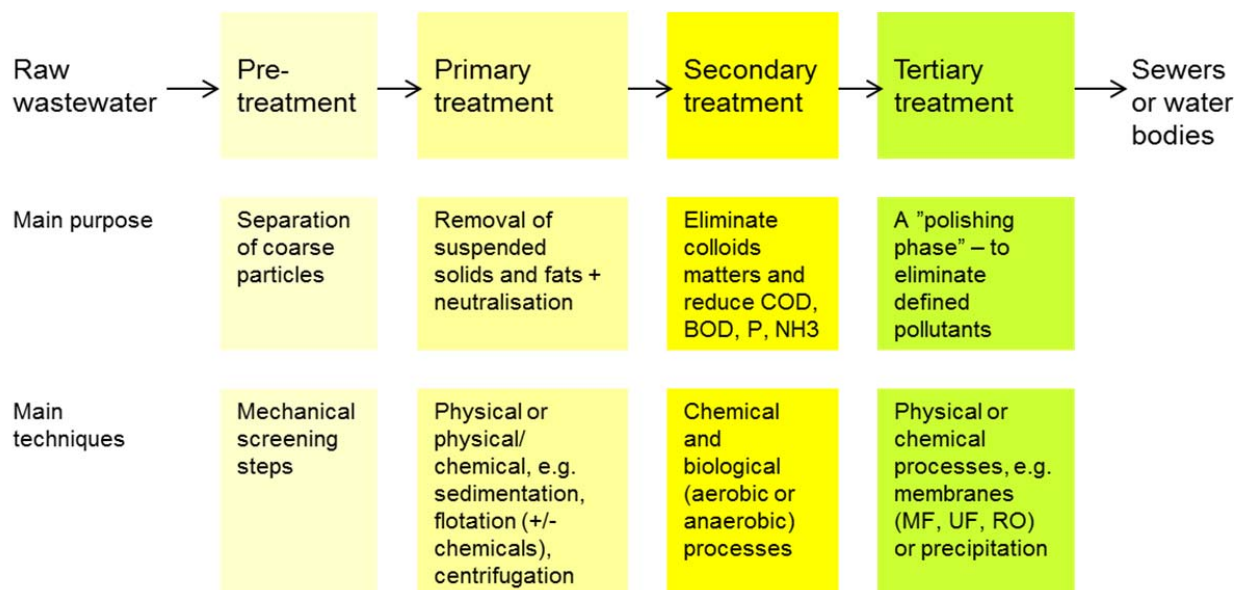


Figure 3.a. Typical treatment process for wastewater from the food industry, with indication of the main purpose and main techniques used in the pre-, primary-, secondary- and tertiary treatments. COD and BOD are chemical and biochemical oxygen demand, respectively. MF, microfiltration; UF, ultrafiltration; RO, reverse osmosis.

3.2. Separation techniques for food processing wastewater

Many of the techniques presented in **Figure 3.a** are classified as separation techniques, in which the constituents of a mixture are separated according to differences in chemical or physical properties such as size, shape, mass, density, or chemical affinity between the constituents. Often one or more of the physical, thermal, chemical or electrical processes available are used alone or in combination (El-Mashad and Zhang, 2007). The following treatment of the wastewater includes physical, chemical, and biological processes to remove

contaminants. The overall aim of the separation and following treatment is to produce an environmentally safe fluid waste stream (treated effluent) and a solid waste (treated sludge) suitable for disposal or reuse, the latter usually as farm fertilizer (Chowhury et al., 2010).

Typical pollutant parameters, of importance to the food industry, are;

- Chemical oxygen demand (COD),
- Biological oxygen demand (BOD),
- Total suspended solids (TSS),
- Fats, oils and greases (FOG), as well as
- Nutrients, mainly nitrogen (N) and phosphor (P).

Along with these parameters the levels of salt and chlorine, for example in the seafood industry, or protein produced as an example by the meat and dairy industries might also be important (Bolzonella et al., 2007).

There are several opportunities of separation techniques for the wastewater generated from the food industry. **Figure 3.b** shows the selection of applied technologies (El-Mashad and Zhang, 2007) of which the major focus in the following sections will be on membrane separation processes (MSPs, such as ultrafiltration) and electro-flocculation as these are the tested methods in the work related to this thesis.

In general, the *physical and thermal* separation processes presented in **Figure 3.b** are effective in reducing the organic load in the wastewaters to levels suitable to discharge into public sewer systems. Some recovery of nutrients, either for recycling or as by-products, may be obtained, however, the selectivity necessary for producing pure products is usually not provided by these methods (El-Mashad and Zhang, 2007). These physical and thermal processes can either (1) separate solids or suspended matter from a liquid by means of gravity or by the differences in gravitational forces; (2) promote the formation of solid particles or the adsorption of molecules from an aqueous solution or a gaseous phase onto a solid surface by means of intermolecular attractive forces; or (3) by thermally changing the stage of the molecules in the wastewater, and hereby e.g. concentrate a particular molecule by evaporating the water. However, no chemicals are added to the water during these processes.

In contrast, the *chemical* separation processes uses chemicals to change the surface characteristics of suspended matter and hereby change the compounds from soluble to insoluble forms which enhances the separation. For instance, by adding surfactant while applying intense stirring to the wastewater, microbubbles are created which will be easy to separate from the bulk liquid phase because of its buoyancy (Sebba, 1987). An example of a well-known and well-used separation method is air flotation, especially dissolved air flotation

(DAF) which is a liquid–solid separation process for liquid suspended colloidal mixtures. DAF involves the dissolution of air at a high pressure in the waste mixture to achieve saturation. By bringing the pressure of the mixture back to atmospheric, the air – in the form of very small bubbles – will rise to the product’s surface carrying with it the colloidal particles which can then be recovered (McDermott, 1976). The DAF process can be improved by adding a chemical flocculant or coagulant (Genovese and González, 1994). This technique is applied at the local herring producer with the collected process waters RSW, SW, PW and SB (**Figure 2.a**). They add chemical precipitation (based on the water flue) along with an acid (based on water pH) to improve the work of the chemicals. Thereafter, they raise the pH again before adding a polymer mixed with air to create flocs that will raise the fat to the surface. On average, they produce around 50 m³ of flocculated fat per week (depending on the fat content of the herring) which is used for biofuel, and the treated water is sent to the community treatment plant for further treatment (Paul Mattsson, 2014).

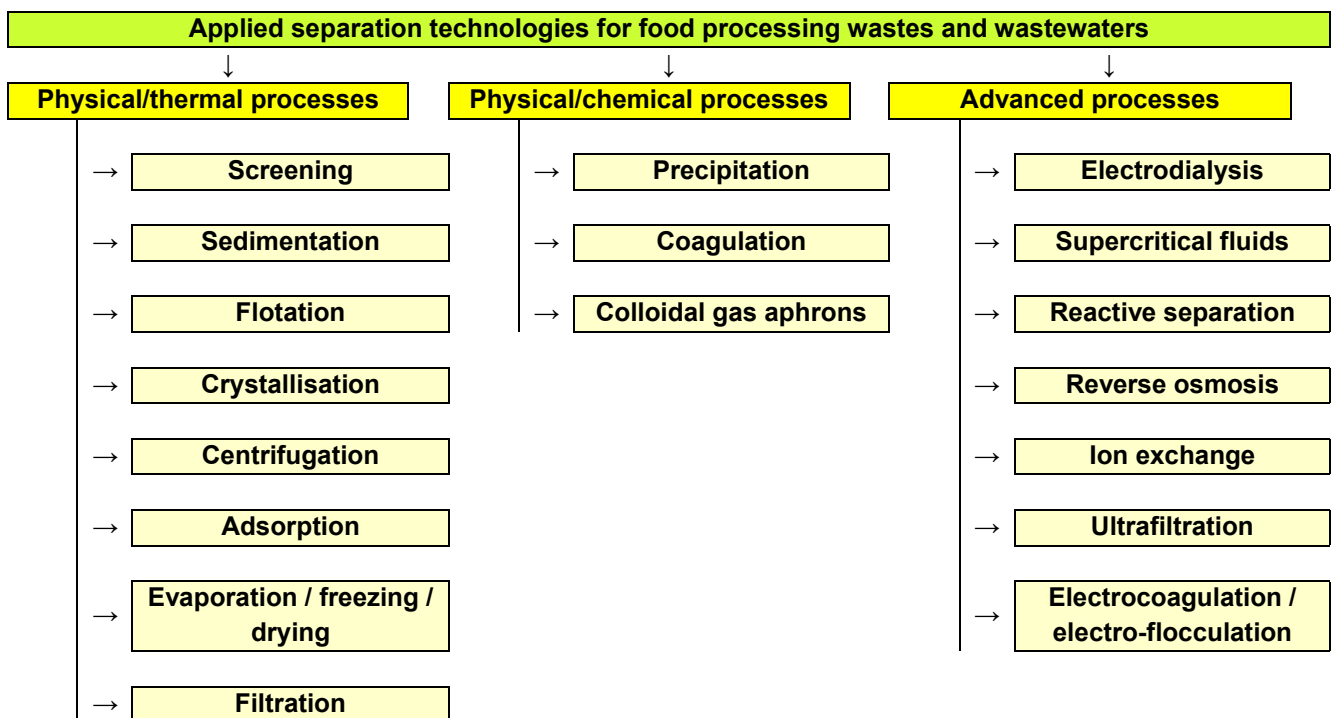


Figure 3.b. Common separation technologies for food processing wastes/wastewaters. Adapted from El-Mashad and Zhang (2007).

Besides these physical, thermal and chemical processes, a number of more advanced separation technologies are applied in treating liquid waste from the food industry. An example is electro-flocculation, a version of electrocoagulation which does not make use of chemicals. As this separation technique is tested in the present work as a part of the recovery treatment for the herring wastewaters an elaboration is provided in the following section.

3.2.1. Electro-flocculation

Electro-flocculation (EF) is an electrochemical pre-treatment method that utilises an electric current by the use of a 'sacrificial' anode that releases iron or aluminium ions through electrolytic oxidation. These coagulant ions lead to aggregation of suspended particulate matter, and as the process also produces hydrogen bubbles the result is flotation of flocs (Ben-Sasson et al., 2011). Some of the reported advantages of EF are that the water is clear, colourless and odourless, that the separation of flocs is easy and that no chemicals are needed (Mollah et al., 2001). Aluminium and iron are used because of their low price, availability and the formation of mainly amorphous metal oxides and hydroxides that have excellent properties for adsorption of soluble species. Compared to iron, aluminium oxides/hydroxides have the advantages of a minimum of solubility at pH between 6 and 7 (Pourbaix, 1974), which indicates the possibility of treating wastewater at neutral conditions.

It has been shown that EF is a time efficient as well as a low-cost technique, for example in a model system consisting of silica particles (Ben-Sasson et al., 2011), in the pre-treatment of microalgae (Lee et al., 2013) and in separating solid and dissolved pollutants from wastewater (Mollah et al., 2001). Ben-Sasson et al. (2011) reported a significant decrease (up to 90%) in filtration-energy consumption in dead-end microfiltration using aluminium-based EF as pre-treatment. Xu et al. (2002) tested aluminium-based EF on the wastewater from egg processing for recovery and utilisation of this by-product. They recovered high concentrations of protein (36-50%) and fat (32-42%) in the generated sludge/flocs. Mollah et al. (2010) used aluminium-based EF to treat a waste stream containing orange II dye, and they reported an efficient removal of more than 94% of the dye under optimal conditions. These results demonstrate the fact that this separation technique may be used successfully in the marinating herring industry.

Another advanced separation technique for use in the treatment of wastewater from the food industry is the various types of membrane separation techniques. These are described in the following sections.

3.2.2. Membrane separation processes

Membrane separation processes (MSPs) has shown to be a good alternative in treating the wastewater from the food industry given that they offer several advantages over more traditional separation processes, such as distillation, crystallization, solvent extraction, etc. Membranes are special devices typically made up of synthetic polymers or inorganic material that creates a barrier which can separate a liquid from a solid stream. They are characterised by their different composition and pore sizes. Generally, they are divided in four sizes; microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). As illustrated in **Figure 3.c** there is no defined transition between the pore sizes, however, it is generally recognised that MF have pore sizes of 0.1-10 μm , UF have pore sizes of 0.01-0.1 μm , NF have pore sizes of 0.001-0.01 μm , and RO have pore sizes less than 0.001 μm (Bolzonella et al., 2007; Coca, 2011). Wastewater treatment in the food industry is commonly carried out by MF and UF, however, if salinity is excessive RO may be of interest (Ditgens, 2007).

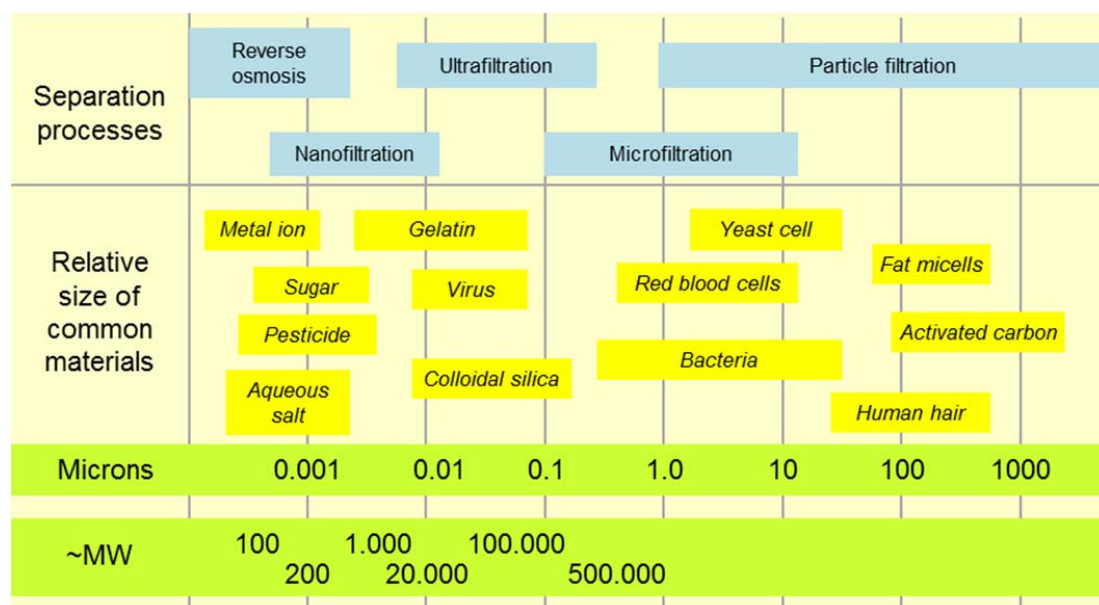


Figure 3.c. Approx. molecular weight (MW in Da) of the compounds separated via reverse osmosis, nanofiltration, ultrafiltration, microfiltration and particle filtration. The approx. cut-off values (in microns, μm) of the membranes are indicated as well together with examples of the relative size of some common materials.

There are several types of membranes. The tubular membranes have been around for a long time and they have a simple design. The main advantages with these membranes are that they can tolerate suspended solids and most notoriously fibers to a very high extent. On the other hand, the disadvantages are that they require a lot of space and energy and changing the membrane may both be difficult and time consuming (Wagner, 2001). Additionally, the trans-membrane pressure (TMP) is the driving force for permeation, and the flux will increase with pressure up to a limiting value depending on the physical properties of the feed to be filtered and the cross-flow velocity. Hence one of the main problems with the membranes is the flux decrease. This decrease is caused by concentration polarisation (CP) and fouling, which are characterised by the unwanted accumulation of material on membrane surfaces and encompass adsorption, gel layer formation, pore blocking, and other processes. The CP results in a rapid drop in flux while the fouling causes a gradual, long-term decay and thus it is common to state the initial flux after a few minutes of filtration (see **Figure 3.d**). An important role in the fouling of membranes is the surface chemical phenomena and this can either be reversible or irreversible. Reversible fouling is mainly caused by solid deposition on the membrane surface and can typically be removed by intermittent hydraulic back flush. The irreversible fouling, however, requires a chemical cleaning which limits the practicability of e.g. UF as a wastewater treatment process (Jönsson and Trägårdh, 1990; Cassini et al., 2011).

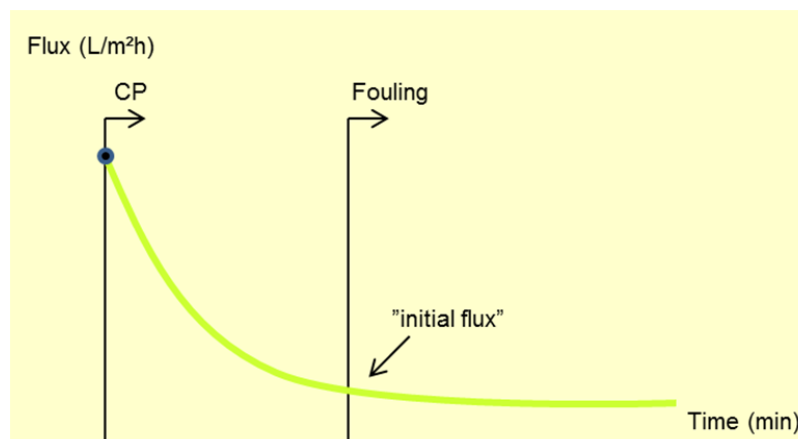


Figure 3.d. Flux ($\text{L/m}^2\text{h}$) decline is caused by concentration polarisation (CP) and fouling. The pure water flux is indicated by (•). Adapted from Jönsson and Trägårdh (1990).

It is common to express the efficiency of a membrane by determining the percentagewise rejection, R , of a given compound. This is done by measuring the concentration of the

component in the permeate and the inlet water and calculate the ratio between these (Afonso and Bórquez, 2002):

$$R = \left(1 - \frac{C_{\text{permeate}}}{C_{\text{feed}}}\right) \cdot 100\%$$

Retention of 100% thus means that the generated permeate is completely free from the compound of interest. Depending on the size of the molecules that is of interest the size of pores in the membrane (MF, UF, NF or RO) is chosen, see **Figure 3.e**. The four filtration types, MF, UF, NF and RO, are each briefly described in the following sections.

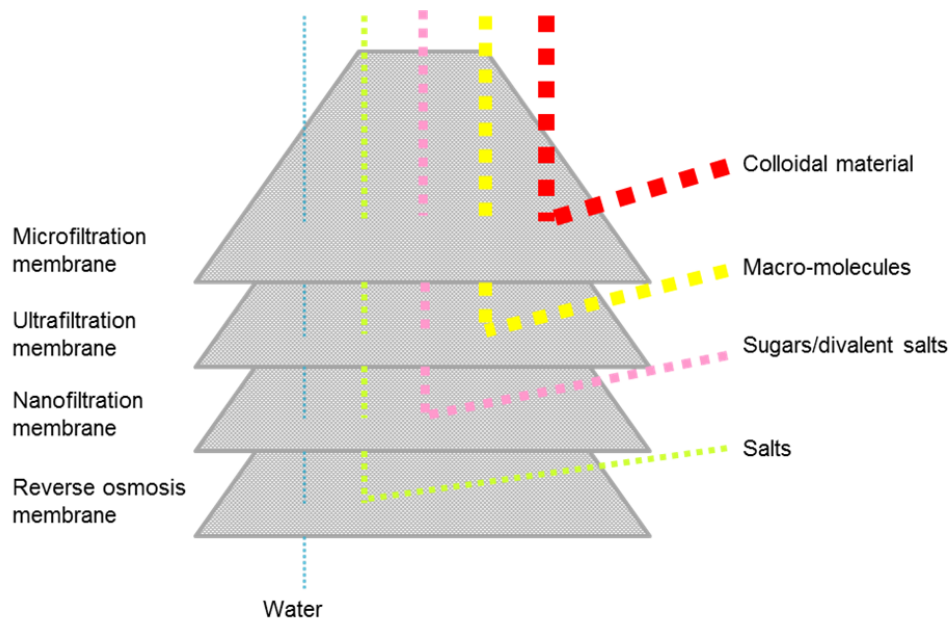


Figure 3.e. Membrane separation. Showing which membrane types allows passage of which molecules.

3.2.3. Microfiltration

Microfiltration (MF) can be used to treat raw and pre-treated food processing wastewater. MF can remove suspended solids, bacteria and large molecules without using any chemicals, while even protein pass the membrane (Wagner, 2001; Baker, 2004), as shown in **Figure 3.c & 3.e**. Although this technology is used frequently in the food industry it should be noted that the main role of the MF membrane is to separate or fractionate wastewater components.

When separating wastewater by MF the hope is to turn the wastewater into a more useful and/or less polluting stream. This technique cannot break down or chemically change any pollutants (Bolzonella et al., 2007).

3.2.4. Ultrafiltration

Ultrafiltration (UF) involves the use of membranes with a molecular weight cut-off (MWCO) in the range of 1–300 kDa and a pore size of $\sim 0.01\ \mu\text{m}$. UF uses pressures greater than 1 MPa and is used to separate high molecular weight (HMW) components like proteins and suspended solids from low molecular weight (LMW) components like mono- and disaccharides, salts, amino acids, inorganic acids or sugars and salts (Salehi, 2014; Wagner, 2001; Baker, 2004). Several studies have reported the use of UF in the recovery of proteins, for instance a 69% protein recovery from fish meal effluents (Afonso et al., 2004). Cassini et al. (2010) demonstrated that the use of ceramic UF in the pre-treatment of isolated soy protein (ISP) wastewater has great potential as they managed to retain 34% COD, 52% protein, and 86% TSS. Such recoveries and organic load retention may allow for additional revenue and a significant reduction of environmental pollutions.

3.2.5. Nanofiltration

Nanofiltration (NF) lies between the separation characteristics of reverse osmosis and UF processes and is widely used for several applications such as water softening and wastewater treatment. With pore sizes in the range of 0.5–1 nm, NF membranes concentrates, fractionates or purifies aqueous solutions of organic solutes with molecular weight between 100 and 1,000 Da and mixtures of monovalent/multivalent salts, and uses pressures between 1 and 4 MPa (Salehi, 2014; Baker, 2004). NF membranes can be used for salt fractionation since the rejection of monovalent salts is lower than that of multivalent salts (Hilal, 2007) and rejection of sodium chloride with NF have been reported to vary from 0-50% depending on the feed concentration (Wagner, 2001). True NF rejects only ions with more than one negative charge, such as sulphate or phosphate, while passing single charged ions, such as chloride. NF also rejects uncharged, dissolved materials and positively charged ions according to the size and shape of the molecule in question, and thus NF can be used for specific separation of very small molecules (Wagner, 2001).

3.2.6. Reverse osmosis

Reverse osmosis (RO) is the tightest possible membrane process in liquid/liquid separation. In principle, water is the only material passing through the membrane and consequently all dissolved and suspended material is rejected (Wagner, 2001), see **Figure 3.e**. RO membranes are characterised by a MWCO of ~ 100 Da, and the process involves pressures 5 to 10 times higher than those used in UF. The pressure is usually between 4 and 10 MPa and particles with molar masses below 350 Da can be concentrated, thus this technique is used to desalinate water (Baker, 2004). Furthermore, this technique is often used to improve the recovery of amino acids present in the UF permeate (Afonso and Bórquez, 2002). The phenomenon of osmosis occurs when a semi-permeable membrane separates a solution from its solvent or a similar solution with a lower concentration. But, the direction of the volume flow can be reversed by applying a hydrostatic pressure to the solution, which has to be higher than the osmotic pressure difference across the membrane (Ditgens, 2007).

Once the size of membrane is decided upon, the type of membrane material should be considered, as the different materials hold different advantages and disadvantages.

3.2.7. Membrane materials

There are many different materials to choose between when selecting a membrane. Cellulose acetate (CA) is the “original” membrane material and is used for RO, NF and UF applications. The main advantage of CA is its low price, and the fact that it is hydrophilic, which makes it less prone to fouling. On the other hand the material has a number of limitations, predominantly with respect to pH and temperature and an inherent weakness of CA is that it can be eaten by microorganisms (Wagner, 2001). Both polysulfone (PSO) and polyvinylidene difluoride (PVDF) are used in UF and MF applications. PSO is practically the only membrane material used in high quantity for a number of food and dairy applications, and this might be due to its exceptional temperature and pH resistance. As a rule, PSO membranes do not tolerate fat, oil, grease and polar solvents, and it has a tendency to become brittle. PVDF is an excellent but rather expensive material. It has good heat stability and is chemically almost as resistant as Teflon (Ditgens, 2007; Wagner, 2001). Polypropylene (PP) is used in MF applications and is chemically a very resistant polymer; however, the temperature stability is limited, and it has a tendency to creep (Wagner, 2001).

Inorganic membranes show a high chemical resistance, which is an extremely important parameter during the washing cycles, considering the likelihood of chemical and biological fouling that can take place. Interactions between protein molecules and the membrane surface and inner pores may occur, resulting in pore blocking, surface deposits and/or gel

layer formation on the top layer, and this will in turn increase the filtration resistance. Still, these interactions are expected to be weaker in inorganic membranes than in organic ones (Afonso and Bórquez, 2002). Also, it is widely acknowledged that hydrophobic membranes have a higher tendency to foul than hydrophilic membranes (Salehi, 2014). The very hydrophilic ceramic membranes can, theoretically, be very effective for MSPs, yet they are extremely expensive.

Although an expensive material, the ceramic membranes made from SiC were pre-selected to be tested in the present work, see **Figure 3.f**. These membranes have recently been certified for use in the food industry, and according to the manufacturer they are extremely hydrophilic, thus very efficient in oil/water separation. Besides this, they are chemically inert and tolerate high salt content and temperature (LiqTech, 2014). Compared with polymeric membranes, ceramic membranes have higher thermal, mechanical and chemical resistance, and when made out of SiC they are extremely stable in harsh conditions for which reason they can withstand repeated aggressive cleaning, steam sterilisation and autoclaving (Zhou et al., 2011; Ciora et al., 2004). As a result, SiC would be well suited for use in food processing such as the herring industry. In a resend study, Hofs et al. (2011) compared the permeability and fouling of four ceramic membranes (Al_2O_3 , ZrO_2 , TiO_2 , SiC) with a polymeric one (polyethersulfone (PES) and polyvinyl-pyrrolidone (PVP) blend), and found that the TMP increased due to fouling; the reversible fouling decreased in the order of polymeric $\approx \text{Al}_2\text{O}_3 \approx \text{ZrO}_2 > \text{TiO}_2 > \text{SiC}$, and for irreversible fouling polymeric $> \text{ZrO}_2 > \text{Al}_2\text{O}_3 > \text{TiO}_2 > \text{SiC}$. Thus the SiC membrane was less prone to fouling than the other materials tested. However, it should be noted that the authors also measured the pore size and found that in general the fouling decreased as the pore size increased, which in the case of TiO_2 and SiC were 5 and 24 times larger than stated by the suppliers, respectively.



Figure 3.f. Silicon carbide membranes used in the present work.

As presented in **Section 3.1** and this section, a variety of separation and treatment methods are available for the wastewater generated from the food processing industry. In the next section some of the applied techniques are presented, with focus on separation methods mainly intended for recovery.

3.3. Applied separation and recovery techniques in the food industry

Depending on the main purpose; *treatment* according solely to environmental considerations or *recovery* of specific components to create an added value, some techniques are more convenient than others. In **Table 3.a** a selection of the currently applied techniques for recovery of (mainly) protein from wastewater in the food industry are presented.

Many of the techniques shown in **Table 3.a** are membrane technologies, which have a great potential for the separation (e.g. concentration, fractionation, and purification) of several types of process waters. For instance, Cassano et al. (2013) studied the combination of two UF processes followed by a NF treatment on olive mill wastewater in order to fractionate and thereby separate organic substances of different molecular weights within this wastewater. They obtained three different fractions; (1) a concentrated solution containing organic substances at high molecular weight (retentate of both UF processes), which could be submitted to an anaerobic digestion for the production of biogas; (2) a concentrated solution (NF retentate) enriched in polyphenolic compounds suitable for cosmetic, food and pharmaceutical industries as liquid, frozen, dried or lyophilized formulations; and (3) a water stream (NF permeate) suitable for reuse in e.g. the olive oil extraction process as process water or in the integrated membrane system as membrane cleaning solution.

In another study, Cassini et al. (2010) demonstrated that the use of UF membranes, in the pre-treatment of ISP wastewater, was very promising for organic load reduction and protein content removal. By use of a ceramic monotubular membrane they were able to recover the very small molecules (8–50 kDa) that were not removed during the production process. This was believed by the authors to possibly result in a large productive and financial advantage for the respective industry. Of the three pore sizes they tested, the smallest, a 5 kDa membrane, gave the best results and showed the least reduction in permeate flux over time (indicating a lower occurrence of CP and fouling phenomena) and the highest retention percentages: 34% COD, 52% protein, and 86% TSS.

Magueijo et al. (2005) have used NF membranes for the recovery of cheese whey organic nutrients, resulting from “Serpa” cheese and curd production. A rich lactose fraction was recovered from the cheese whey through NF and the permeate fraction was mainly process water and salt. The water recovery was approximately 80% thus concentrating the cheese

Table 3.a. Applied separation and treatment techniques for wastewater in the food industry

Method	Product	Main purpose	Ref.
MF +UF	Fish meal	Recovery and concentration of protein	Afonso et al., 2004
UF	Fish brine	Recovery and fractionation	Paulson et al., 1984
	Fish meal		Afonso and Bórquez, 2002
	Soy protein	Recovery and concentration of protein	Cassini et al., 2011; Cassini et al., 2010
	Surimi		Miyata, 1984; Dumay et al., 2008
	Fish	Concentration of protein and lipids	Mameri et al., 1996
UF + NF	Olive mill	Purification of bioactive compounds and reuse of water	Cassano et al., 2013
NF	Low-alcohol beer	Reduction of alcohol %	Salehi, 2014,
	Cheese whey	Recovery of lactose	Magueijo et al., 2005
RO-NF	Fruit juice	Improve the concentration step	Nabetani, 1996
RO	Fish meal	Pre-concentration of solids	Matthiasson and Sivik, 1978
Electro-osmosis	Apple pomace, and vegetable	Dewatering the waste to increase the solid content	Orsat, 1996
Foam separation	Soy whey	Recovery of protein	Wang et al., 2013
Two-stage foam separation	Whey	Recovery of protein	Jiang et al., 2011
Microscreen unit + sequencing batch reactor	Swine manure and fish cannery	Cleaning of the wastewater	Fernandes et al., 1991
Solid phase extraction	Beer	Purification of bioactive compounds	Barbosa-Pereira et al. 2013
Decanter + anaerobic & aerobic treatment	Tuna	Treatment of wastewater	Achour et al., 2000
Microalgae-containing microbiota	Rainbow trout	Treatment of wastewater	Riaño et al., 2011
Deammonification and anaerobic carbon removal	Fish	Energy-efficient treatment of wastewater	Trautmann et al., 2011
Evaporation + biological treatment	Olive oil	Separation and concentration	Vitolo et al., 1999

whey nutrients five times. NF has also been used to meet the growing demand for low-alcohol and alcohol-free beer, and 8-10 times reduction in the alcohol concentration, while retaining the beer flavour, have been reported (Salehi, 2014).

In the concentration of whey a coupling of UF and RO have been extensively used and have allowed for the development of a broad array of whey protein concentrates (WPC). Among the promising applications for MSPs in whey processing are those aimed at increasing the protein content of WPC, fractionating whey protein, and improving the specific functional properties of whey protein (Rosenberg, 1995). As well as in the whey protein industry the MSPs in the fish industry are often applied both for adding value of by- and co-products, and for the treatment of process water. By applying the MSPs, potential added value is recovered along with an effluent clean-up, which could help solving the environmental problems by complying with the increasingly strict legislation worldwide concerning industrial pollution (Afonso and Bórquez, 2002).

In this PhD, the tested separation techniques were applied on wastewater generated from a herring industry. The potential in separating fish wastewater to recover both protein and lipids are not entirely new. However, to our knowledge the work regarding the brines generated during the marinated herring processing are far from completed. As an example of a related study, Afonso et al. (2004) studied an integrated process of MF pre-treatment followed by UF and concluded that this is a promising technique for the recovery and concentration of protein from fish meal effluents as they were able to recover 69% of the protein. Such recoveries allows for a revenue rise and a significant reduction of environmental pollutions. Matthiasson and Sivik (1978) have shown the use of a RO membrane in the application of treating the water from fish filleting machines, and the concentrate generated could then be applied in e.g. soups, while the permeate could be reused in the process. UF has also been used for the separation and concentration of water-soluble protein from wastewater from fish processing plants. For instance, Miyata (1984) showed that about 90% of the protein from the feed could be concentrated with a tubular cellulose acetate membrane. However, in contrast to these promising results Afonso and Bórquez (2002) concluded that the protein rejection (3-39%) they obtained with a mono-tubular mineral UF membrane of 15 kDa MWCO was insufficient compared to their goal; environmental pollution abatement and recycling of water and protein from the fish meal industry.

In a study by Dumay et al. (2008), four membrane materials (PSO, PVDF, polyacrylonitrile (PAN), and regenerated cellulose) at five different MWCOs (ranging from 3 to 100 kDa) were tested in the treatment of washing waters from surimi manufacturing. The results showed that UF, especially with a 10 kDa regenerated cellulose membrane, seemed to be an appropriate technology for the recovery of protein and lipids from sardine surimi wastewater with a reduction of COD by a factor of four. These results confirmed those obtained by Mameri et al.

(1996) where UF reduced the level of BOD₅ by about 80% for fishery washing water. In general, the amounts of fish processing wastewater are high and mainly consist of protein and lipids (Palenzuela-Rollen et al., 2002), and thus testing different (combinations of) separations techniques for these waters to recover the protein, lipids and other potential bioactive compounds are of high interest for the fish industry. By proper treatment, the fish industry could create revenue on the recovered molecules along with lowering the expenses for discarding the polluted wastewater.

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4. Bioactive compounds in fish processing waters

Bioactive compounds cover a variety of molecules; protein, peptides, several amino acids as well as a large number of phenolic-based compounds (Karel et al., 1966). During the processing of food, vast amounts of both solid and liquid waste are generated and whenever these wastes contain bioactive compounds, strategies for recovery and utilisation should be considered. In **Chapter 3** a selection of technologies for treatment and separation of liquid waste were presented. This chapter presents the bioactive compounds present in liquid waste from the fish industry, and thus the bioactive compounds which could be expected to be present in the brines generated during the final ripening period of the marinated herring products investigated in this thesis.

The food-derived bioactive compounds are mainly peptides and they represent a source of health-enhancing components. These bioactive peptides are found in many plants, milk, meat, and fish of various kinds, and are usually 2-20 amino acids residues in length. Most often they are inactive within the sequence of their parent protein but they can be released during gastrointestinal digestion or during food processing (Erdmann et al., 2008). Currently, fish protein hydrolysates (FPH) are considered the most important source of protein and bioactive peptides. Some studies have reported the use and application of protein hydrolysates in human nutrition (Clemente, 2000; Neklyudov et al., 2000) while others have reported the biological activity of various FPH *in vivo* and *in vitro* by means of their bioactive peptides (Duarte et al., 2006; Je et al., 2004; Liaset et al., 2009). FPH are usually produced by adding commercial available enzymes which degrade the fish protein. However, during the production of marinated herring, it has been shown that proteases from the herring intestine and muscle tissue participate in the proteolytic degradation of the muscle protein (Nielsen, 1995; Stefánsson et al., 2000). This enzyme activity leads to an increase in soluble nitrogenous compounds, such as peptides and amino acids (Nielsen, 1995; Nielsen and Børresen, 1997), and thus it is the herring's own enzymes from the viscera that cause an increase in LMW compounds in the fillet, which is believed to influence the final sensory characteristics of the product (Nielsen, 1995). Furthermore, the brines generated during the long ripening period might be just an important source of protein and bioactive peptides as the FPH. Moreover, there is a possibility that the enzymatic activity is still present in the brine after the marinating process is completed, thus these enzymes could as well be regarded as a valuable product.

Today, considerable amounts of protein-rich by- and co-products from the seafood industry are discarded, or processed into fish oil, fish meal, fertilizer, pet food and fish silage (Penven et al., 2013). These generated by-products create a cost burden for the seafood industry in terms of waste disposal and only minor benefits generated, as these recycled products

possess low economic value. The still growing demand for a sustainable use of fish protein co-products (FPCP) has led to the development of processes for the recovery and hydrolysis of protein, the assessment of their functionalities, and application into different products (He et al., 2013). Recent studies have identified a number of bioactive compounds from residual fish muscle protein, fish oil, fish bone, internal organs and shellfish and crustacean shells (Je et al., 2005a; Kim et al., 2001), and generally, a far better profitability is obtained by producing human consumables. The highest profitability is expected from bioactive compounds such as functional foods or nutraceuticals (Kim and Mendis, 2006).

Functional foods might be defined as *“those foods or ingredients that when consumed regularly produce a specific beneficial health effect beyond their basic nutritional properties”* (Gil-Chávez et al., 2013). Normally, these foods contain different amounts and types of bioactive compounds (Roberfroid 2002; Nobili et al., 2009; Bernal et al., 2011) and before the product can be called a functional food, the claimed health benefit for the human organism has to be demonstrated. Examples of beneficial effects of bioactive compounds are: decrease in cholesterol levels, alleviation of lactose intolerance, maintaining remission of Crohn's disease, faster relief from diarrhea, and inhibition of cancer cell proliferation *in vivo* and *in vitro* (Hekmat et al., 2009; Nobili et al., 2009; Marette et al., 2010).

Nutraceuticals, on the other hand, might be defined as *“dietary supplements that deliver a concentrated form of bioactive compounds from food, present in a non-food matrix, and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods”* (Shahidi, 2009). They are normally consumed in pharmaceutical presentations such as pills, capsules, tablets, powders or vials (Espín et al., 2007). They are usually isolated or synthesised from pure chemicals from natural sources and their efficiency have to be clinically proved to prevent or cure the target disease.

No matter if the bioactive compound is intended for use as a functional food ingredient, a new food product or as a nutraceutical, the activity must be described/defined and tested. Today, the primary used sources of bioactive peptide originate from dairy products. However, the marine environment represents a number of protein resources including algae by-products and FPCP, and the environment in which they are found makes these resources a new and relatively untapped source for bioactive compounds (Rustad and Hayes, 2012). Furthermore, the fat content in fish which in general vary from 2– 30%, depending on the type of species, dietary, geographic, environmental, reproductive and seasonal variations, is mainly composed of two types of fatty acids; eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both EPA and DHA are n-3 LC-PUFAs (Kim and Mendis, 2006), which are known for their prevention from atherosclerosis (von Schacky, 2000), reduction of blood pressure (Appel et al., 1993), benefit for diabetic patients (Sheehan et al., 1997), among many suggested nutraceutical potentials. Apart from the high-quality fat, fish contains protein, minerals,

vitamins and antioxidants that are also believed to impact human health (Simopoulos, 1997; Elvevoll and Österud, 2003), and several of these molecules share the common property of interfering with the lipid oxidation in some way.

4.1. Lipid oxidation

Lipid oxidation is of great concern to the consumers and the food industry because it deteriorates flavour, colour, and nutritional quality of foods and leads to the development of undesirable odours and potentially toxic reaction products (Lin and Liang, 2002). Furthermore, cancer, coronary heart disease, atherosclerosis, arthritis, diabetes and Alzheimer's diseases have been reported to be caused in part by oxidation or free radical reactions in the body (Sarmadi and Ismail, 2010; Bougatef et al., 2010; Diaz et al., 1997), and thus prevention of lipid oxidation is an important issue.

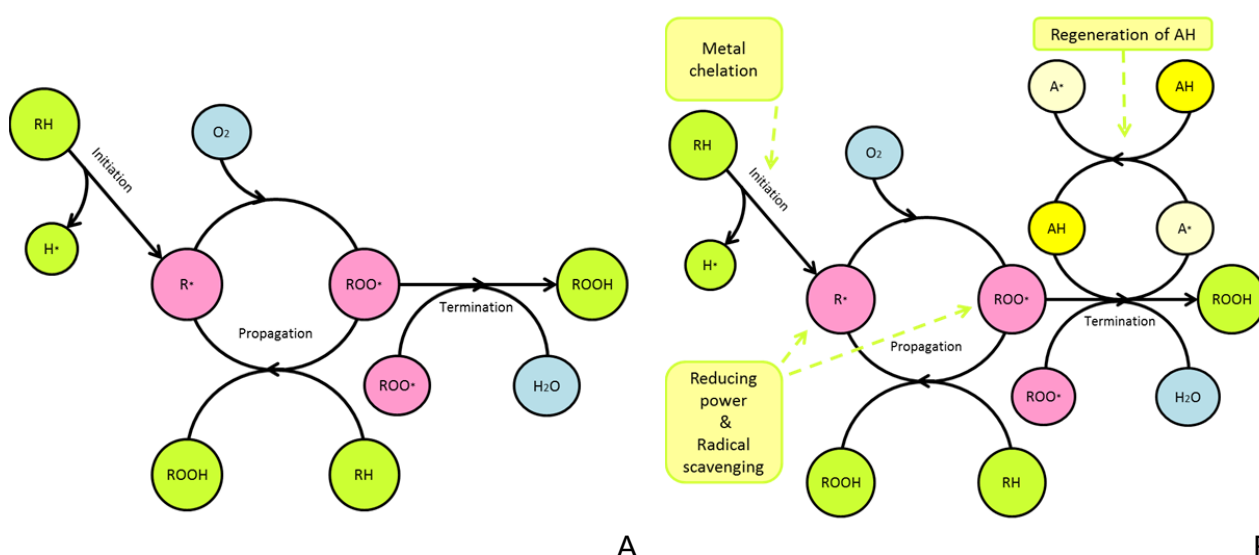


Figure 4.a. Lipid oxidation (A) and the antioxidants effect on lipid oxidation (B). **A)** The stable (lipid) molecule RH is turned into an unstable radical (R•) through initiation. This radical can further react with atmospheric oxygen and become a reactive oxygen species (ROO•) which can further react with other RH. This propagation is continued until two radicals are reacting with each other and creating a water molecule and a (lipid) hydroperoxide (ROOH). **B)** Antioxidants (AH) can work as metal chelators by affecting the metals that are prone to initiate the oxidation. Other antioxidants have the ability to reduce radicals or scavenge them from further reaction. These AH react with the radicals (R• or ROO•); creating a RH or ROOH and a A•, which is less unstable than R•. Some AH affects the oxidation indirectly by regenerating other AH which directly leads to termination.

Lipid oxidation is a complex process of free radical-mediated chain reaction which involves three stages; initiation, propagation and termination (see **Figure 4.a-A**), and which creates reactive oxygen species (ROS) that can negatively affect membrane lipids, protein and DNA (Pampanin et al., 2012). Heat, light, oxygen, enzymes, transition metals, metalloproteins and microorganisms can act as initiators or promoters for the process. Among the methods employed for preventing lipid oxidation, addition of antioxidants is the most effective, convenient and economical strategy by stabilising food and non-food products (Wanasundra and Shahidi, 2005; Decker et al., 2005). Many synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (**Figure 4.b**), which are both powerful synthetic antioxidants, are used as food additives and although they show stronger antioxidant activities than those of natural antioxidants (e.g. α -tocopherol and ascorbic acid) the use of these has begun to be restricted due to their believed carcinogenic activity (Ito et al., 1986). In recent years, the interest in finding new antioxidants from natural sources, which have little or no side effects, to replace the synthetic antioxidants has been great (Mendis et al., 2005). Furthermore, this has been one of the motivating factors in the present project, as the herring brine might represent a natural source of antioxidants that could hold the ability to reduce the oxidative damage in foods.

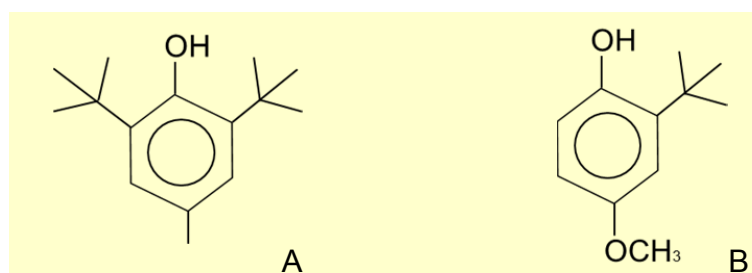


Figure 4.b. Structure of butylated hydroxytoluene, BHT (**A**) and butylated hydroxyanisole, BHA (**B**).

Lipid oxidation may be delayed or prevented by antioxidants through different mechanisms, e.g. radical scavenging, reduction of ROS, chelation of metal ions, and regeneration of other antioxidants, see **Figure 4.a-B**. The next section elaborates on the different types and sources of antioxidants.

4.2. Antioxidants

Antioxidants can act in different ways, and according to their mode of action, they may be classified as radical terminators, chelators of metal ions, or oxygen scavengers that react with oxygen in closed systems (see **Figure 4.a-B**). Primary antioxidants react with high-energy lipid radicals to convert them to more thermodynamically stable products, thus acting as radical scavengers. Secondary antioxidants, also known as preventive antioxidants, function by hindering the rate of chain initiation by breaking down hydroperoxides (Shahidi and Naczki, 2004).

When antioxidant peptides possess metal chelation or hydrogen/electron donating activity, they can interact with free radicals and terminate the radical chain reaction or prevent their formation (Ren et al., 2008). It has been recognised that transition metal ions, such as Fe^{2+} and Cu^{2+} are involved in many oxidation reactions *in vivo* by catalysing the generation of ROS (Stohs and Bagchi, 1995), and thereby are implicated in cardiovascular and neurological diseases, such as atherosclerosis, and Alzheimer's and Parkinson's diseases (Blat et al., 2008). Thus chelating agents that decrease free iron, and other transition metal ions, may possess therapeutic potential (Torres-Fuentes et al., 2012).

The overall antioxidant activity of the bioactive peptides is dependent on the amino acid residues and the sequence, and it has been shown that the activity can be enhanced by the presence of hydrophobic amino acids and one or more residues of histidine, proline, methionine, cysteine, tyrosine, tryptophan and phenylalanine (Je et al., 2007; Ren et al., 2008; Karel et al., 1966; Marcuse, 1960; 1962). The two aromatic amino acids, tyrosine and phenylalanine, are known to act positively as direct radical scavengers, which is most likely due to the special capability of phenolic groups to serve as hydrogen donors (Jun et al., 2004). The amino acids such as histidine, methionine and cysteine are also very important to the radical scavenging activity of peptides. In case of histidine, it is the imidazole group that has the proton-donation ability and thus makes it a strong radical scavenger (Yong and Karel, 1978). Methionine is prone to oxidation of the methionine sulfoxide, and cysteine can donate the sulphur hydrogen (Bougatef et al., 2010; Hsu et al., 2009). Kawashima et al. (1979) have reported the antioxidative activity of peptides having branched-chain amino acids, such as valine, leucine and isoleucine, and Chan and Decker (1994) claimed that peptides containing basic amino acids (arginine, lysine and histidine) are electron acceptors, which thus take electrons from radicals formed during the oxidation of unsaturated fatty acids. Peña-Ramos et al. (2004) have reported a relationship between the antioxidant properties of the peptides and the presence of aromatic and imino acids. The presence of hydrophobic sequences in peptides could interact with lipid molecules and thereby scavenge by donating protons to lipid derived radicals (Je et al., 2007). By interfering with the formation of reactive radicals (alkoxyl or peroxy), both at the initiation and propagation phase, scavengers function as radical chain

breaking antioxidants. In non-polar systems, this effect seems to be mediated by donation of a hydrogen atom; however, in aqueous media one-electron transfer is more likely to occur (Jovanovic et al., 1996).

Consequently, the radical scavenging activity of peptides is most likely due to the hydrogen atom donor activity of the phenolic and indolic groups, as a higher stability is obtained in the phenoxyl and indolyl radicals (**Figure 4.c**) compared to that of simple peroxy radical, ROO* (Pihlanto, 2006). Although the antioxidant mechanism of peptides is not yet fully understood, there seems to be a relationship between the antioxidant properties of peptides and the presence of aromatic, imidazole and sulphur-containing amino and imino acids (Shahidi and Zhong, 2010). In a study by Pampanin et al. (2012) the small (<10 kDa) natural bioactive peptides from herring by-products, such as skin and residual material, were investigated, and they reported several peptides with motifs that had cardiovascular system activity (50%), antioxidant activity (40%) and immunomodulatory activity (8%). Motifs with antioxidant activity were e.g.: AH, DLYA, EL, GPPGPPGPP, GPPGPPGPPG, LQGM, MY, SLYA, QGAR, VW. The most represented motifs were the AH and EL. The dipeptide MY from sardine muscle have also been shown to be antioxidative (Erdmann et al., 2006) and the tripeptides LKP, IKP and LRP from fish muscle have been shown to have antihypertensive effect (Nagai et al., 2006). **Table 4.a** lists some known peptide sequences with antioxidant activity.

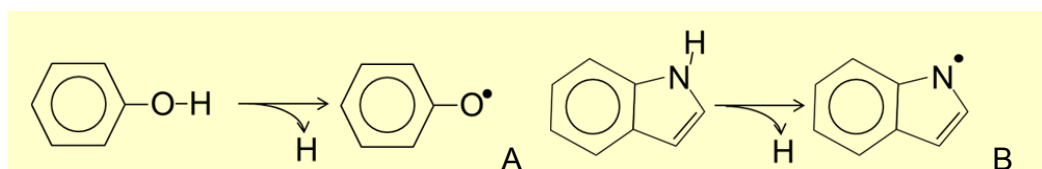


Figure 4.c. Structure of a phenolic group and the corresponding phenoxyl radical (**A**), and an indolic group and the corresponding indolyl radical (**B**). Both the phenoxyl radical and the indolyl radical are more stable than the peroxy radical, ROO*.

Table 4.a. Peptide sequences with antioxidant activity

Sequence	Origin	Ref.
AH, DLYA, EL, GPPGPPGPP, QGAR, GPPGPPGPPG, LQGM, MY, SLYA, VW,	Herring by-products	Pampanin et al. (2012)
MY	Sardine muscle	Erdmann et al. (2006)
LKP, IKP, LRP	Fish muscle (antihypertensive)	Nagai et al. (2006)
LHY	FPH from sardinelle	Bougatef et al. (2010)
GPHypGPHypGPHypGPHypG, GGHypGPHypGPHypGPHypGPHypG	FPH from Alaska pollack skin	Kim et al. (2001)
LPHSGY	FPH from Alaska pollack frame	Je et al. (2005b)

In general, the phenolic acids are reported as radical scavengers, with the efficiency of the antioxidant depending on number, position and nature of the substituting groups. The most general feature related to high activity is reported to be the number of neighbouring hydroxyl groups which make gallic acid (2,4,5-trihydroxy-benzoic acid, **Figure 4.d**) one of the most potent antioxidants (Chimi et al., 1991; Rice-Evans et al., 1996). The activity of phenolic compounds as antioxidants arises from the low dissociation enthalpy of the ArO–H (Ar, aromatic ring structure) bond, which renders them high in hydrogen atom donation capacity and hybrid resonance of the phenyl ring that helps stabilise the corresponding phenoxyl radicals (**Figure 4.c-A**) (Shahidi and Zhong, 2010). There have been more than 8,000 different phenolic constituents identified, ranging from simple single-hydroxylated phenols to highly polymerised compounds (Harborne, 1993). Most phenolic compounds are water-soluble due to the hydrophilicity arising from their hydroxyl groups. Hence their functional properties are seen mainly in aqueous environments or water compartments in body tissues. Consequently, the poor lipophilicity of phenolics results in a limited bioavailability after oral ingestion, which is caused both by low absorption and rapid elimination in the body (Shahidi and Zhong, 2010).

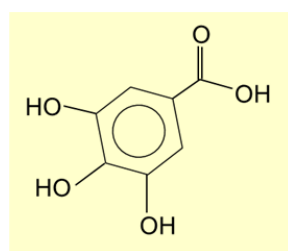


Figure 4.d. Structure of gallic acid, 2,4,5-trihydroxy-benzoic acid.

An important note is that although the antioxidant properties of peptides and phenolics are due to metal ion chelation, free radical scavenging or electron donation, Chen et al. (1998) have demonstrated that none of these properties can be correlated solely with the antioxidant activity of the compounds. This implies that the overall antioxidant action is most likely attributed to the cooperative effects of the mechanisms mentioned. Thus, in the experimental work conducted on the herring brine in this project, the presence of all three antioxidants properties were analysed to evaluate the overall antioxidant activity.

When reviewing the literature for *bioactive compounds in fish processing waste/ wastewater*, the majority of the work is done on solid waste (e.g. Liceaga-Gesualdo and Li-Chan, 1999; Sathivel et al., 2003; Je et al., 2005b; Samaranayaka and Li-Chan, 2008; Bougatef et al., 2010). For instance, in a study by Sannaveerappa et al. (2007a) the antioxidative effect of herring light muscle press juice against haemoglobin-mediated oxidation of washed cod

mince during ice storage was tested. After fractionation of the press juice into LMW (<1 kDa) and HMW (>1, >3.5 and > 50 kDa) fractions, the antioxidant effect were compared with that given by endogenous levels of two antioxidant candidates: ascorbic acid and uric acid and the oxidation were followed by determining rancid odour, peroxide value, and redness. It was concluded that native herring light muscle press juice had a strong antioxidative activity and that a major part of the activity lies in the LMW (<1 kDa) fraction. Yet, a common strategy is to change the functional properties of the protein from FPCP by enzymatic hydrolysis, thereby decreasing the molecular weight, increasing the number of ionisable groups and causing exposure of hydrophobic groups (Panyam and Kilara, 1996). Considering the long ripening period in the production of marinated herring, and the fact that the traditional barrel-salted versions are marinated as whole-headed herring, and therefore with their endogenous enzymes, this liquid waste is thought to display some of the same antioxidative qualities as herring FPH.

4.2.1. Fish protein hydrolysates

Protein hydrolysates are breakdown products of enzymatic conversion of native protein into smaller fragments of peptides, thereby making hydrolysates the most available amino acid source for various physiological functions of the human body (Neklyudov et al., 2000). The enzymatic hydrolysis results in a decrease in peptide size, which in turn can modify the functional characteristics of the peptides and improve their quality (Petersen, 1981). The functional properties of FPH may be improved by the use of specific enzymes and by choosing a defined set of hydrolysis conditions, such as time, pH, and temperature (Hall and Ahmad, 1992). Liceaga-Gesualdo and Li-Chan (1999) found that by using a commercial endopeptidase preparation, Alcalase, they were able to easily transform underutilised herring into FPH. The functionality tests conducted on the FPH indicated that the emulsion stability was improved in the hydrolysate as compared to the unhydrolysed herring. As for the foamability, the hydrolysate provided a higher foam expansion; however, it was unable to maintain foam stability over time.

In a study by Sathivel et al. (2003), the functional, nutritional, and antioxidative properties of hydrolysed whole herring and herring by-products (body, head and gonad) were evaluated. They concluded that the antioxidative activity of whole herring FPH was highest (about half of the activity of BHA and BHT), followed by that of body, gonad, and head. Furthermore, the whole herring FPH contained 84.4% protein and all of the herring protein hydrolysates were reported to meet or exceed the essential amino acid requirements for adult humans, hence this study demonstrated that FPH derived from herring or its by-products may serve as a good source of desirable quality peptides and amino acids. Besides the antioxidative activity

and the high protein content, the herring FPHs also had desirable colour, solubility, fat absorption, and emulsification stability.

Antioxidant activity has been reported for FPH from various fish protein sources such as tuna cooking juice (Hsu et al., 2009), yellowfin sole frame (Jun et al., 2004), Alaska pollack frame (Je et al., 2005b), and pacific hake muscle (Samaranayaka and Li-Chan, 2008). In **Table 4.a** some antioxidant active sequences are presented. One example is Bougatef et al. (2010) who have studied the antioxidant potential in sardinelle by-products protein hydrolysates obtained using various proteases. They reported that the sardinelle FPHs were found to possess good antioxidant activity, and the highest antioxidant activity was found in the hydrolysate obtained with a crude extract from sardine viscera. Moreover, they found that the tri-peptide Leu-His-Tyr displayed the highest radical-scavenging activity. Kim et al. (2001) have hydrolysed gelatine extracted from Alaska pollack skin, and they obtained a FPH which consisted of peptides ranging from 1.5 to 4.5 kDa and showed high antioxidative activity. Two peptides were isolated, which were composed of 13 and 16 amino acid residues, see **Table 4.a**. Both peptides contained a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp. The 16 amino acids peptide was reported to be a natural antioxidant which had potent antioxidative activity. Dong et al. (2008) used two commercial available enzymes, Alcalase and Flavourzyme, to hydrolyse Silver carp, and they two found that the LMW peptides were responsible for the observed antioxidant activity in the hydrolysates. In another study, Je et al. (2005b) identified an antioxidative peptide, Leu-Pro-His-Ser-Gly-Tyr (672 Da), from Alaska pollack frame protein hydrolysate that was prepared with mackerel intestine crude enzyme and reported that the isolated peptide showed high scavenging activity on hydroxyl radicals.

It is important to keep in mind, that FPHs are the results of hydrolysing solid by-products or whole unused fish, and thus the activities found in herring FPH cannot be expected to be present to the same extend in the brines generated during the production of marinated herring. However, the brines are still thought to display some of the same qualities as herring FPH. Additionally, there is a notable difference between herring FPH and the brines generated during the production of marinated herring; in the former, commercial enzymes are used to obtain the hydrolysis whereas in the brine the endogenous enzymes from the herring are sufficient in cleaving the protein into smaller peptides and amino acids.

The fish viscera, mainly stomach, pyloric caeca, and intestine (see **Figure 4.e**), is an important source of proteases that helps cleaving larger protein into smaller peptides (Bezerra et al., 2001, 2005; Souza et al., 2007; Espósito et al., 2009; Silva et al., 2011). These enzymes are an important tool for the food industry due to their ability to transform raw materials into improved food products. For example, proteases are used to hydrolyse protein and polypeptides to produce LMW peptides and free amino acids, thus increasing the digestibility of the product (Shahidi and Kamil, 2001). Moreover, many of the enzymes exhibit

high catalytic activity at relatively low concentrations (Haard, 1998). Considering the specific characteristics of these enzymes, fish processing by-products are currently used for enzyme extraction. A range of proteolytic enzymes including pepsin, trypsin, chymotrypsin and collagenases are commercially extracted from marine fish viscera in large scale (Byun et al., 2003; Kim et al., 2002; Park et al., 2002). For instance, pepsin from cod and collagenase from crab hepatopancreas are used as a gentle way to remove fish skin and membranes from roe sacs and other products, and a cold-adapted enzyme from cod is commercial available as a fermentation aid in the fermentation of matjes herring (Simpson and Haard, 1984). Raksakulthai et al. (1986) have reported that due to the broad array of proteinases and peptidases in squid hepatopancreas, the enzymes from this by-product have found their use in reducing the fermentation time of fish sauce from about 2 years to only 4 months.

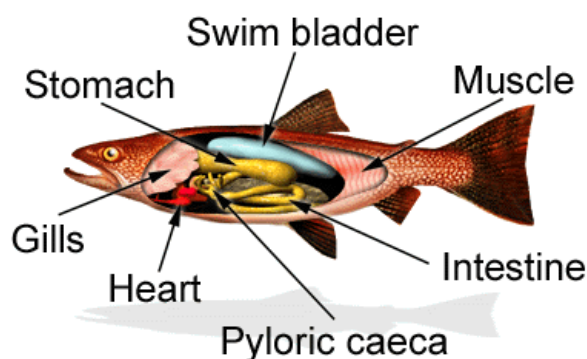


Figure 4.e. A schematic view of the internal anatomy, showing e.g. stomach, pyloric caeca and intestine, which are the important sources of fish viscera proteases (adapted from <http://www.arctic.uoquelp.ca>).

The discussion in this section indicates that herring FPH have been studied and reported to be a valuable source of peptides with antioxidant activity as well as a source of amino acids. Furthermore, the herring viscera is a source of proteolytic enzyme, which during the ripening process of marinated herring are involved in the degradation of the muscle protein into smaller peptides that potentially are a source of natural antioxidants. Currently, the richest sources of natural antioxidants come from higher plants and their constituents. Shahidi and Zhong (2010) reported fruits, vegetables, spices, herbs, cereals, grains, oilseeds, leguminous seeds, teas, coffee and cocoa as the major sources of plant-derived antioxidants. In the spice-cured versions of the marinated herring products (SC and TSp, **Chapter 2**), the initial brines consist of salt, sugar and spices (unknown mixture). A broad variety of spices and herbs has been evaluated for their antioxidant capacity (Madsen and Bertelsen, 1995;

Yanishlieva et al., 2006). For instance, rosemary, sage, oregano, thyme and black pepper have been reported to contain bioactive phenolic compounds working as antioxidants and chances are that some of these spices are a part of the spice mix used in the production of the spice-cured herring. The following section discusses the antioxidant activity of phenolic compounds expected to be present in the wastewaters from the marinated herring industry.

4.2.2. Phenolic compounds

Bioactive phenolic compounds are widely distributed in nature and are the most abundant antioxidants in the diet e.g. in components of fruits, vegetables, and some beverages. Due to the worldwide trend to avoid or at least reduce the use of synthetic additives, such as BHT and BHA (**Figure 4.b**), there is a need to identify natural sources of food antioxidants (Ito et al., 1986). Thus, the ability of phenolic substances, including flavonoids (e.g. flavones, isoflavones or anthocyanins, **Figure 4.f-A-C**) and non-flavonoids (e.g. phenolic acids, **Figure 4.f-D**, respectively), to act as antioxidants has recently been extensively investigated for their chemistry, sources, antioxidant activity and health effects (Katalinic et al., 2006; Rice-Evans et al., 1996; Shahidi, 2009; Wong et al., 2006; Zhong et al., 2007).

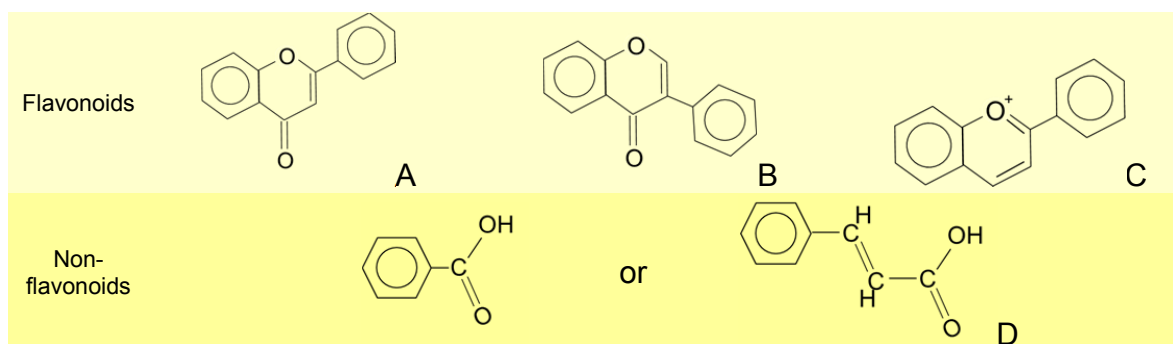


Figure 4.f. General structure of three types of flavonoids; **(A)** flavone, **(B)** isoflavone, **(C)** anthocyanin, and **(D)** two types of phenolic acids. All the structures can have various side chains, -R, (not indicated).

Yanishlieva et al. (2006) have reviewed natural antioxidants from herbs and spices. Rosemary is one of the most effective spices widely used in food processing, and is today commercially available for use as an antioxidant in Europe and in the United States. Sage is used in foods for flavouring and seasoning and is, together with rosemary, one of the spices

with the best antioxidant activity. Furthermore, its extracts are well known as efficient antioxidants (Djarmati et al., 1991; Pizzale et al., 2002; Cuvelier et al., 1996; Takacsova et al., 1995). Oregano is very often used as a spice and its flavour is highly favourable to consumers all over the world. It is also valued for its antimicrobial and antioxidant properties. In a study by Kikuzaki and Nakatani (1989) five different phenolic compounds were isolated from a methanol extract of oregano and all five showed antioxidative activity. Thyme contain phenolic hydrocarbons (thymol and carvacrol) that are known to inhibit lipid oxidation and *p*-cumene-2,3-diol which is known to be a strong antioxidant (Yanishlieva et al., 2006). The antioxidative activity of black pepper can, at least partially, be ascribed to the presence of glycosides of the flavonoids kaempferol, rhamnetin and quercetin (Vösgen et al., 1980) as well as to phenolic amides (Nakatani et al., 1986).

In spite of scientific documentation of the antioxidative effect of many spices and herbs, it is still mainly extracts from leaves of rosemary and sage that are used as antioxidative spice additives. A range of commercial products containing extracts of rosemary are available; some of the products are water dispersible, others are oil soluble, and in order to exploit the synergistic effect, some of them are combined with tocopherols (Yanishlieva et al., 2006). The spice composition in the marinated herring brines are, as previously mentioned, confidential and thus we can only guess which spices and herbs there might be added to the spice-cured herring products. If they contain these known antioxidative spices, their activity might still be present in the used brines, and thus could be recovered and utilised as antioxidant additives.

Whether the herring brines studied in this work contain proteins or peptides with antioxidant activity, free amino acids, active enzymes or phenolic compounds originating from the spices added, these bioactive compounds could offer an added value to the food industry. Simultaneously, they could be regarded as a resource for the herring producers rather than the expense it nowadays is, paying for discarding this heavy organic loaded wastewater. The antioxidative bioactive compounds could have potential for nutritional, pharmaceutical, cosmetic and nutraceutical application as functional ingredient due to their health promoting effects, and if they are as active as the FPH derived from various fish species they could be used as replacement for the synthetic antioxidants, BHT and BHA (Hsu, 2010; Bougatef et al., 2010; Chalamaiah et al., 2012). However, before being used for human consumption, the effectiveness of these bioactive compounds should be studied and documented safe in both animals and humans (Chalamaiah et al., 2012). In addition, antioxidants for use in food processing must be inexpensive, nontoxic, effective at low concentrations (0.001–0.02%), capable of surviving processing (carry-through), stable in the finished products, and devoid of undesirable colour, flavour and odour effects (Shahidi and Zhong, 2010).

The studies presented in this chapter clearly show the potential in the brines generated during the ripening period of marinated herring; both the possibility of the presence of phenolic

compounds, due to the spices, and of the presence of endogenous enzymes, in TSp and TSa brines. In the next chapter, **Chapter 5**, the experimental work is presented in the form of five scientific papers.

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5. Own findings

The experimental work performed throughout this PhD were planned in order to answer the working hypothesis presented in **Chapter 1**.

Based on the known knowledge presented in **Chapter 2, 3 and 4**, the working hypothesis could be theoretically accepted meaning that literature study show that it is a potentially valid hypothesis. However, experimental work is needed and based on the reviews in these chapters the objectives and aims for the experimental work was planned. The work is presented in the five papers, Paper A, I, II, III and B;

- A. In Paper A, four process waters were characterised and the recovery of the proteins and fatty acids was tested with electro-flocculation and ceramic ultrafiltration.
- I. In Paper I, six different brines were characterised for proximate composition, antioxidant and enzymatic activity. All the brines were generated during the final ripening step, from barrel to glass jar, in the production of marinated herring.
- II. Paper II aimed at testing the ceramic ultrafiltration membranes in combination with electro-flocculation or a 50 µm pre-filter, on two of the most interesting brines, based on the results from Paper I. These technologies were evaluated for their applicability and efficiency in treating the herring effluents.
- III. In Paper III, the LMW compounds (<10 kDa) from three brines were fractionated into 94 fractions and all analysed for the protein/peptide content, total phenolic compounds and antioxidant activity. From each type of brine three pooled samples were selected for characterisation of amino acids and phenolic acids and for the identification of the peptide sequence.
- B. Paper B tested three brines for their ability to work as natural antioxidant sources, both at native pH and at pH 2 and 11. The brines were tested as coating agents for frozen herring and as additives in fresh mince herring.

A general discussion of my own findings is presented after the papers in **Chapter 6**, followed by the main conclusions and perspectives in **Chapter 7**.

PAPER A

Osman, A.; Gringer, N.; Svensson, T.; Yuan, L.F.; Hosseini, S.V.; Baron, C.P.; Undeland, I. (2015). Quantification of biomolecules in herring (*Clupea harengus*) industry process waters and their recovery using electroflocculation and ultrafiltration. *Food and Bioproducts Processing*, 96, 198

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Quantification of biomolecules in herring (*Clupea harengus*) industry processing waters and their recovery using electroflocculation and ultrafiltration

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ABSTRACT

Four types of herring industry processing waters; refrigerated sea water (RSW), storage water (SW), processing water from cutting (PW) and pre-salting brines (SB) were subjected to chemical characterization and biomolecule recovery using electroflocculation (EF) and ultrafiltration (UF). The highest protein and fatty acid content were found in SB's, up to 12.7 ± 0.3 and $2.5 \pm 0.1 \text{ g L}^{-1}$, respectively. Long chain *n*-3 polyunsaturated fatty acids represented up to 44.5% of total fatty acids. In all samples, leucine and glutamic acid/glutamine were the dominating amino acids while calcium and magnesium were the dominating trace elements. EF plus UF in series recovered up to 80% proteins and fatty acids from SB's and reduced chemical oxygen demand by 70%. Foaming and emulsifying properties of biomolecules were improved or unaffected by EF/UF treatment. To conclude, large amounts of biomass are currently lost per ton of processed herring, e.g. $\sim 9.2 \text{ kg}$ proteins and $\sim 4.1 \text{ kg}$ fatty acids; EF/UF represents a promising way of turning such losses to a potential income.

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1. Introduction

Atlantic herring (*Clupea harengus*) is a small fatty pelagic fish, found along the coastlines of the northern parts of the Atlantic Ocean (FAO, 2012). It is rich, for example, in long chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFAs), vitamin D and high value proteins, and its consumption is linked to improved health benefits (Lindqvist et al., 2009). In Northern Europe,

there is a long tradition of producing and consuming marinated herring. The industrial production of marinated herring yields considerable side streams of process water since each ton of final product requires up to 7 m^3 water. The largest water consumption takes place in the early steps in the production chain; from the fishing step to the start of the maturation in barrels. This is depicted in Fig. 1 along with the different types of generated process waters. The first type of water used on

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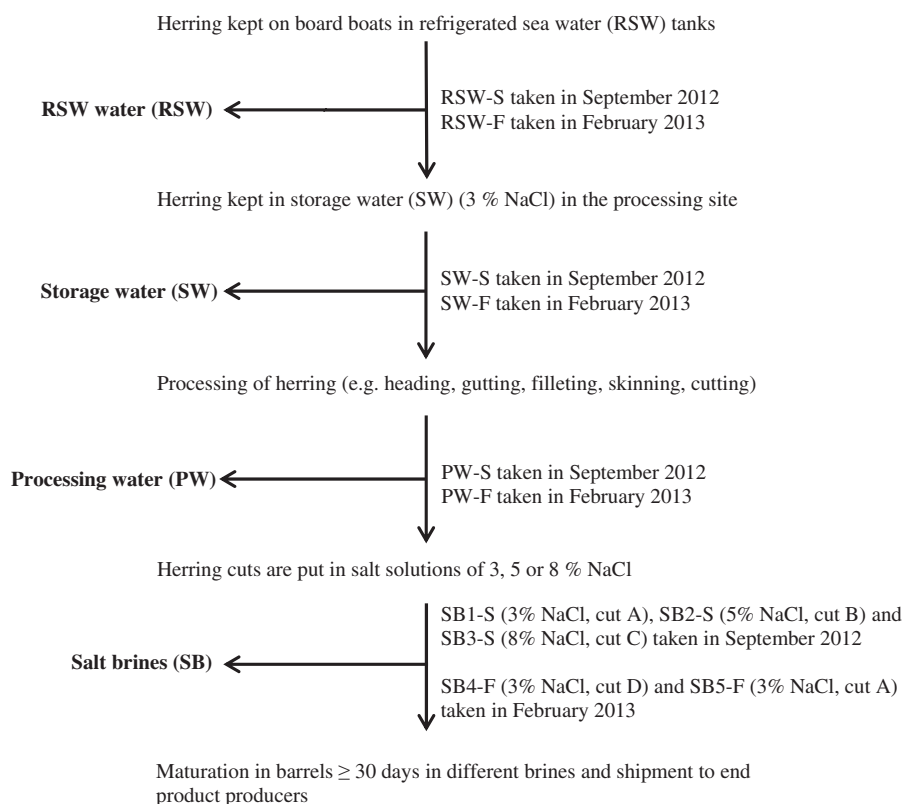


Fig. 1 – A schematic representation of the different types of process waters generated during the early stages of producing marinated herring. To the right, the process water samples taken out in this study are shown. Note that the muscle exposure of the different cuts used increases in the order A–D.

board vessels is refrigerated sea water (RSW). Upon landing, herring is stored until further processing in tanks containing 3% salt solution; here considered as storage water (SW). Herring is then processed into different cuts and a processing water (PW) is generated. Subsequently, herring cuts are placed in salt brine (SB) with 3–8% salt before it is matured in different types of marinating brines for at least 30 days at 2–4 °C until final processing steps take place.

The substantial leaching of biomolecules from the herring to the described water types raises their levels of organic matter, and thus, their chemical and biological oxygen demand, i.e. COD and BOD. The latter are the basis for tariffing for the discharge of process water. Today, some marinated herring industries reduce the BOD and COD of effluents by separating solid organic pieces using sieves, and by the application of chemical flocculants such as polyaluminium chloride in floatation units. Chemical flocculation, however, renders the flocculated biomass unsuitable for further use as food or feed. To the best of our knowledge, only herring marinating brines have so far been the subject for compositional analyses (Andersen et al., 2007; Christensen et al., 2011; Gringer et al., 2014; Svensson et al., 2004; Szymczak and Kołakowski, 2012; Taheri et al., 2014; Drost et al., 2014). Thus, the total amount of biomolecules lost by leaching throughout the entire herring processing chain remains unknown.

Implementation of modern, non-destructive and environmentally friendly technologies, such as ultrafiltration (UF), for the concentration of valuable marine biomolecules in process waters is anticipated to further increase the competitiveness of the marinated herring industry (Drost et al., 2014). Within some other segments of the seafood industry, the potential of using UF for the concentration of biomolecules wasted in process water has already been highlighted. For instance, it has

been reported that 60–97% of total proteins and up to 90% of total lipids from different types of process water generated in the cuttlefish, shrimp, sardine surimi and fish meal processing industries were recovered by UF (Afonso et al., 2004; Amado et al., 2013; Benhabiles et al., 2013; Dumay et al., 2008). In recent years, novel ceramic membrane materials made from silicon carbide have been certified for use in the food industry. According to the manufacturer, these membranes have the characteristics of being very hydrophilic with high tolerance over a wide range of chemical and physical conditions such as high salt content and extreme pH values and therefore seem suitable for herring industry process waters.

Besides UF, interesting developments have also been made in the field of aggregation/flocculation, aiming to avoid the use of chemical flocculants in order to improve environmental sustainability and allow potential use of the collected biomass within the food/feed industries (Tsai et al., 2002; Valero et al., 2011). One such example is electroflocculation (EF), which applies an electric current on electrolytes passing through a flocculation unit which consists of an anode and cathode. This process produces hydrogen bubbles which contribute to the floatation of the flocs which, in turn, are produced by coagulant ions, generated as a result of the electric current applied to the anode and cathode (Tsai et al., 2002; Valero et al., 2011). Reported advantages of EF include the ease of operation and separation of flocs, the good quality of the produced flocs and the fact that no chemicals are added to aid in flocculation (Tsai et al., 2002; Valero et al., 2011).

The aim of this study was to chemically characterize the four side streams of herring marinating process water described in Fig. 1 (i.e. RSW, SW, PW and SB) and apply silicon carbide membrane technology either alone or in combination with EF, on a pilot scale to concentrate interesting

marine biomolecules currently lost in these water streams. The concentrating trials were also followed by measuring COD values and by brief characterization of protein functionality in concentrates. To our knowledge, no separation and recovery attempt of marine biomolecules from the addressed herring process water has been previously reported.

2. Materials and methods

2.1. Sampling the different side streams of processing water

Herring was caught in September 2012 (samples denoted as -S) in Kattegat, and in February 2013 (samples denoted as -F) in Skagerrak. Sampling of the four different types of process water, arising during the processing of these two herring catches, was performed on-site at a local herring producer (Paul Mattsson AB, Ellös, Sweden). All samples are shown in Fig. 1. For RSW-S and RSW-F, the samples were taken upon the arrival of the boat to the processing site, which was 5 and 20 h after catch, respectively. For SW-S and SW-F, samples were taken after 0, 12, 24, 36, 48, 72 and 96 h of herring storage. PW-samples were only taken once, at the end of the cutting line, which was completed in few minutes. The types of herring cuts produced are named A, B, C and D; with the area of direct muscle exposure (i.e. without the skin or guts acting as barriers) toward water or salt brine increasing in the order A–D. For PW-S and PW-F, samples were taken during the production of cuts B and D, respectively. SB samples were taken after 0, 5, 10, 15, 20 and 25 h of incubating specific herring cuts in the different salt solutions. The SB samples taken in this study represented several combinations of herring cuts and salt concentrations (3%, 5% or 8%) depending on the company production schedule. The SB samples collected in September included cut A in 3% salt (SB1-S), cut B in 5% salt (SB2-S) and cut C in 8% salt (SB3-S). The SB samples collected in February included cut D in 3% salt (SB4-F), and cut A in 3% salt (SB5-F). All collected samples were stored for up to 2 days at -20°C in the processing site, transported to the laboratory in cooler bags with ice packages and finally stored at -80°C until analysis. All samples were analyzed, in triplicate, for pH, dry matter, protein content and total fatty acid content. Some of the samples (RSW-S/-F, SW-S/-F (48 h incubation), PW-S/-F and SB1-SB5 (15 h incubation)) were also selected for the analysis of total amino acids, polypeptide profile and trace elements; these were RSW and SW incubated for 48 h, as well as PW and SB's (1–5) incubated for 15 h.

2.2. Dry matter, pH and ionic strength

Five mL of each sample were weighed in dry and pre-weighed glass tubes and put in an Electrolux 939 oven (Electrolux, Stockholm, Sweden) at 60°C for 24 h and then at 105°C until a constant weight was obtained. Dried samples were then cooled in a desiccator and the dry matter was determined as follows:

$$\% \text{ Dry matter} = [(W3 - W2)/W1] \times 100 \quad (1)$$

where W3 is the weight of the sample and the tube after drying, W2 is the weight of the empty tube following a pre-drying step and W1 is the weight of the sample before drying.

The pH of the samples was determined at 20°C using pH M210 standard pH meter (Radiometer analytical, Lyon, France)

and a calibrated Hamilton double pore electrode (Bonaduz, Switzerland).

To standardize the protein functionality testing, the ionic strength (IS) was analyzed using a standard conductivity meter (Radiometer analytical, Lyon, France) and was converted to % NaCl using a standard curve.

2.3. Total proteins and polypeptide profile

The protein content of all samples was analyzed by the bicinchoninic acid assay (BCA assay) (Thermo scientific, Göteborg, Sweden) using bovine serum albumin (BSA) as a standard. For selected samples, the polypeptide profile was also evaluated by SDS-PAGE (Laemmli, 1970); all gels and reagents were from Bio-Rad (Solna, Sweden). Electrophoresis was conducted using Mini-protean TGX 4–20% pre-cast gels. Approximately $10\mu\text{g}$ of protein, were mixed with $5\mu\text{L}$ Laemmli sample buffer and $1\mu\text{L}$ 2-mercaptoethanol, heated at 95°C for 5 min, cooled and then loaded into the wells. Tris/glycine gels were run at 200 V for 40 min. The protein marker was a broad range standard (6.5–200 kDa) and staining was done with Coomassie blue. The gels were scanned using a GS-800 calibrated densitometer. Qualitative analysis of the bands was done using Quantity one 4.5.1 software (Bio-Rad, Solna, Sweden).

2.4. Total amino acids

Total amino acids analysis was performed on selected samples as described by Farvin et al. (2010). Briefly, $500\mu\text{L}$ of each sample were subjected to microwave-assisted acid hydrolysis in sealed ampoules. Following filtration through $0.2\mu\text{m}$ filters, derivatization was carried out using the EZ: Fast kit from Phenomenex A/S (Allerød, Denmark). Analysis of amino acid derivatives was then conducted by liquid chromatography–mass spectroscopy (LC-MS); the full details of the method can be found in Farvin et al. (2010). It should be stated that this procedure did not allow the detection of methionine, tryptophan and cysteine.

2.5. Total fatty acids

Fatty acid analysis was performed after extraction according to Lee et al. (1996) and subsequent methylation according to Lepage and Roy (1986) with some modifications. Identification and quantification of the fatty acids was carried out by gas chromatography–mass spectroscopy (GC-MS). Margaric acid C:17 was used as an internal standard and the ratio between chloroform and methanol used in the extractions was 1:2. Following phase separation, the chloroform was collected, transferred into a new tube and then evaporated until dryness under nitrogen at 30°C using a TurboVap LV evaporator (Zymark Corporation (Hopkinton, USA)). The dried residual was dissolved in 2 mL toluene. Then, 2 mL of 10% (v/v) acetyl chloride in methanol were added and the mixture was incubated for 2 h at 70°C . One mL of Milli-Q water and 2 mL of petroleum ether were added to the tubes, which were vortexed for 20 s and then centrifuged at $1000 \times g$ for 5 min. The upper organic phase was collected and evaporated under nitrogen at 40°C . Evaporated samples were then dissolved in 1 mL isooctane, from which $200\mu\text{L}$ were used for GC-MS analysis of methylated fatty acids. The analyses were run on an Agilent Technologies 7890A GC system connected to Agilent Technologies 5975 C inert MSD (Kista, Sweden) as described elsewhere (Cavonius et al., 2014). Total fatty acids

were calculated as the sum of all measured fatty acids with the internal standard removed. The ratio between total fatty acids, measured by GC–MS, and total fat, measured gravimetrically following chloroform/methanol extraction, has earlier been set to around 0.8:1 (Undeland et al., 1997).

2.6. Trace elements

For analysis of copper, iron, zinc, nickel, cobalt and manganese, the method by Fredrikson et al. (2002) was used. In short, samples were subjected to acid microwave-aided digestion where after ion chromatography (IC) analyses were conducted. Calcium and magnesium analysis was performed on digested samples using atomic absorption spectroscopy (AAS). The system consisted of an Ultra AA boosted lamp supply, a 240 FS AA lamp chamber, a high intensity Ultra AA coded Multi-element lamp Al/Ca/Mg, an SPS 3 auto-sampler and a flame ionization system consisting of mark 7 spray chamber and mark 7 air/acetylene burner; all parts were from Agilent Technologies (Kista, Sweden). The time measurement was 2 s, and the read delay was set at 20 s. The lamp current was 10 mA and the final concentration of cesium chloride/lanthanum chloride in the sample, prior to analysis, was 10% (v/v). Quantification of calcium and magnesium was conducted using calcium and magnesium atomic absorption standards from Ultra Scientific (Rhode Island, USA).

2.7. Concentrating biomolecules using electroflocculation (EF) and ultrafiltration (UF) by ceramic membranes

The different types of process water used for the concentration trials were SB produced from cut D incubated for 15 h in 3% salt (trial 1), SB produced from cut B incubated for 20 h in 5% salt (trial 2) and PW produced during the preparation of cut C (trial 3). All three concentration trials were conducted on a pilot scale (100 L) on-site using silicon carbide (SiC) membrane technology as the main concentration step; and, in trial 1, with EF used as a pre-treatment step. In trial 1, a thin fat layer was also observed on top of the SB, which was manually removed by a sieve with a pore size of 1 mm, prior to the EF pre-treatment step. For EF in trial 1, a custom-made unit was provided by A-Factory (Hørsholm, Denmark). The flow rate of the process water into the EF unit was 1000 mL min⁻¹ and the voltage applied to the electrodes was 200 V. The foam/floc produced as a result of EF was manually collected (full recovery was however not possible since particles were stuck to the equipment), while the outlet of EF was first filtered through a 50 µm polypropylene filter and then treated by UF using the high flux CoMem asymmetric SiC membrane (25 mm × 305 mm) with channel dimensions of 3 mm, a pore size of 0.04 µm and a membrane area of 0.09 m², provided by LiqTech International (Ballerup, Denmark). In trials 2 and 3, EF was not used and the selected process water were directly filtered through a 50 µm polypropylene filter and then treated by UF using the same membrane as above. In all the above trials, the transmembrane pressure (TMP) was 1.4 bars, the cross flow was 2–3 m/s and the temperature of the different types of process water at the start of the UF process was 10–12 °C. During the UF-treatments, the temperature increased to 18 °C in trial 1, to 34 °C in trial 2 and to 38 °C in trial 3. Concentration factors in each trial were calculated by dividing inlet volume by concentrate volume. All collected samples (floated fat layer, EF foam, UF inlet and UF outlets) were stored at –20 °C in the

processing site for up to 2 days, transported to the laboratory in cooler bags with ice packages and kept at –80 °C until further analysis.

2.8. Chemical oxygen demand (COD)

Samples from the concentration trials were analyzed for their COD values, according to Himebaugh and Smith (1979) with slight modifications. Briefly, 2.5 mL of each sample, after appropriate dilution, were added to a screw capped glass tube (20 mm × 15 mm). Then, 2.5 mL of the digestion solution (4.9 g of potassium dichromate, 16.7 g of mercuric sulfate and 83.5 mL of concentrated sulfuric acid prepared in 500 mL deionised water) and 3.5 mL of sulfuric acid–silver sulfate solution (5 g of silver sulfate prepared in 500 mL sulfuric acid) were added to the sample. The glass tubes were gently inverted three times and then placed in a heating block set at 150 °C for 2 h. Following this, the glass tubes were cooled under cold running water and the absorbance of the produced chromic ions was measured at 605 nm. Aliquots of potassium hydrogen phthalate solution, corresponding to 100–1500 mg L⁻¹ COD, were used to create a standard curve.

2.9. Functionality testing

Foaming capacity (FC) and stability (FS%) of starting brines and concentrates from trials 1–2 were analyzed using the shaking method (Hammershøj et al., 1999). Emulsifying activity and stability index (EAI and ESI, respectively) were determined according to Pearce and Kinsella (1978). Sunflower oil and protein solution at a ratio 1:3 were homogenized by a Polytron homogenizer (Luzern, Switzerland) at 18,000 rpm for 1 min. Immediately and after 10 min, emulsion aliquots were mixed with 0.1% sodium dodecyl sulphate (SDS) and the absorbance at 500 nm was measured. Protein concentrations of samples in the FC, FS%, EAI and ESI tests were matched against the most diluted sample (5.9 mg protein mL⁻¹). In order not to change the native IS (here converted to % NaCl) of the samples, dilutions were done in NaCl solutions of the same concentration as the respective sample (in trial 1: 1.31–2.03%, in trial 2: 3.24%). BSA at 5.9 mg protein mL⁻¹, and at an IS/pH average to each trial (in trial 1: 1.79%/6.58 and in trial 2: 3.24%/6.52, respectively), was used as a reference.

2.10. Statistics

All analyses were performed in triplicate unless otherwise stated. Results are expressed as mean values ± standard deviation (SD). Regression analysis was performed in order to estimate the effect of time on the content of proteins and total fatty acids. ANOVA was performed in order to analyze whether there were significant differences between the different samples. All calculations were done using Excel 2010™. Significant differences were defined at $P < 0.05$.

3. Results

3.1. pH and dry matter

The pH of RSW (-S and -F) and PW (-S and -F) was 6.9 ± 0.1 and 7.0 ± 0.05 , respectively, while the pH of SW-S and SW-F varied between 6.7 and 6.8 over the 96 h sampling period. The pH of SB samples was slightly more acidic; 6.5–6.7, 6.3–6.7, 6.4–6.7,

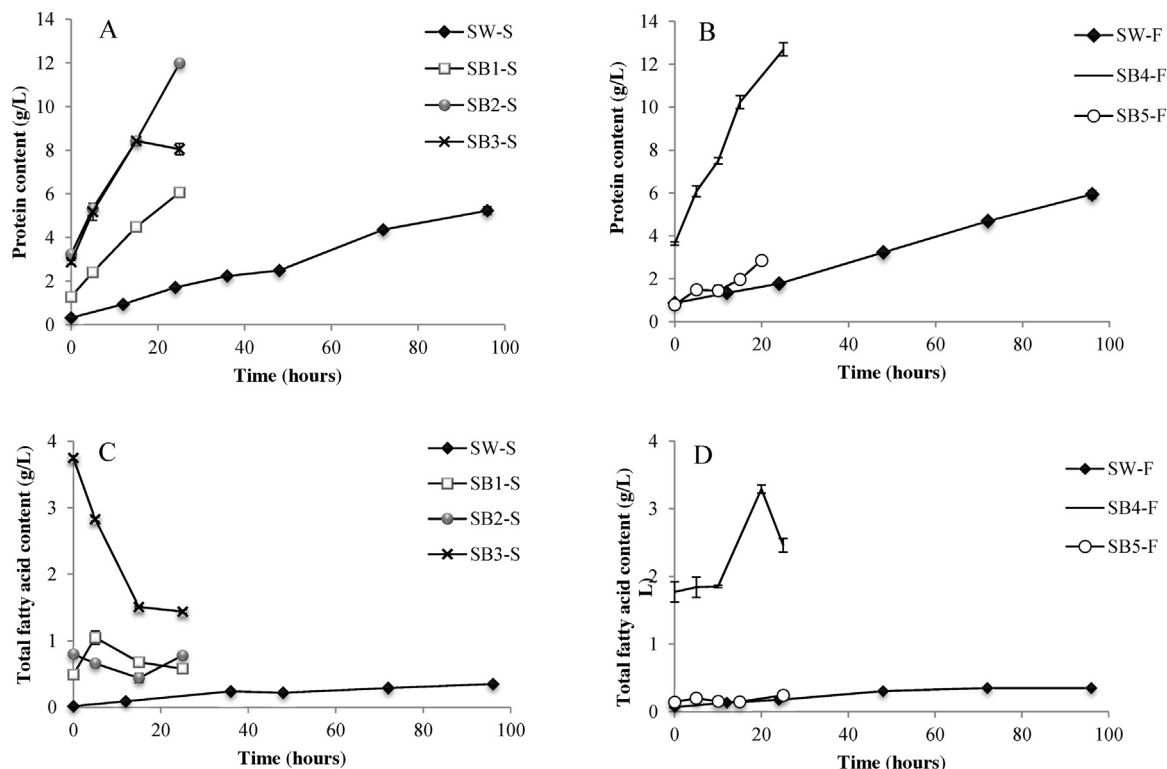


Fig. 2 – Content (g L^{-1}) of proteins (A, B) and total fatty acids (C, D) as a function of time in the different SW and SB obtained in the fall (A, C) and the spring (B, D). Explanations to the sample codes are seen in Fig. 1.

6.3–6.5 and 6.5–6.7 for SB1-S, SB2-S, SB3-S, SB4-F and SB5-F, respectively, over the 25 h sampling period. The incubation time and the different catching occasions did not systematically affect the pH of SW and SB samples.

The lowest values of dry matter content were obtained in PW samples, i.e. 1.15 ± 0.05 and $0.44 \pm 0.04\%$ (w/w) for PW-S and PW-F, respectively. For RSW-S and RSW-F, the dry matter content was 1.45 ± 0.02 and $2.54 \pm 0.02\%$ (w/w), respectively. In SW-S and SW-F, it ranged between 2.8 and 3.8% (w/w), taking into account the different incubation times (≤ 96 h). Compared to these types of process water, the dry matter of SB samples was higher; 2.9–3.7, 3.2–5.6, 6.5–8, 3.5–5 and 2.9–4.2% (w/w) for SB1-S, SB2-S, SB3-S, SB4-F and SB5-F, respectively, over the 25 h sampling period. Incubation time had no significant effects on the dry matter of SW samples ($P > 0.05$). For SB samples, the dry matter tended to increase as a function of time, but this tendency was only significant ($P < 0.05$) for SB4-F.

3.2. Total proteins

The lowest protein content was found in RSW-S and RSW-F, i.e., 0.33 ± 0.01 and $1.06 \pm 0.02 \text{ g L}^{-1}$, respectively. Corresponding values for PW-S and PW-F were 1.3 ± 0.05 and $3.4 \pm 0.1 \text{ g L}^{-1}$, respectively, while those obtained for SW-S and SW-F after 96 h incubation were 5.2 ± 0.2 and $5.9 \pm 0.2 \text{ g L}^{-1}$, respectively (Fig. 2a and b). Compared to the above samples, higher protein contents were obtained in SB samples at the end of incubation; for instance, $12 \pm 0.1 \text{ g L}^{-1}$ in SB2-S (Fig. 2a and 2b). The protein content increased significantly ($P < 0.05$) as a function of incubation time for all SW-samples and for the SB1-S, SB2-S and SB4-F samples (Fig. 2a and b). The protein content made up a substantial percentage of the dry matter in most of the tested process waters, with the highest percentage ($30.1 \pm 0.9\%$, w/w) obtained in PW-S.

3.3. Total fatty acids

The lowest total fatty acid content was obtained in RSW-S and RSW-F, i.e. 0.017 ± 0.001 and $0.15 \pm 0.007 \text{ g L}^{-1}$, respectively, while the corresponding values in PW-S and PW-F were 1.93 ± 0.14 and $0.93 \pm 0.07 \text{ g L}^{-1}$, respectively. For both SW-S and SW-F, the total fatty acid content reached $0.35 \pm 0.01 \text{ g L}^{-1}$ after 96 h incubation (Fig. 2c and d) and incubation time had a significant effect ($P < 0.05$) of fatty acid levels in these samples. In SB samples, on the other hand, the highest content of total fatty acids was, in most cases, not obtained at the end of incubation (Fig. 2c and d). Consequently, incubation time here had no significant effect on the total fatty acid content. In line with the data shown in Fig. 2, the contribution of total fatty acids to the dry matter of the tested process waters was significantly ($P < 0.05$) lower compared to the contribution of proteins; at the most it contributed to $21.2 \pm 1.7\%$ (w/w) in PW-S.

3.4. Fatty acid profile

Saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) were observed in the tested process waters. In general, the dominant fatty acids were myristic acid C14:0, palmitic acid C16:0, palmitoleic acid C16:1 (n-7), oleic acid C18:1 (n-9), gadoleic acid C20:1 (n-9), eicosapentaenoic acid EPA C20:5 (n-3), cetoleic acid C22:1 (n-11) and docosaheptaenoic acid DHA C22:6 (n-3) (for full fatty acid profiles, see supplementary material Tables 1 and 2). Other fatty acids, such as stearic acid C18:0, vaccenic acid C18:1 (n-7), linoleic acid C18:2 (n-6), α -linolenic acid C18:3 (n-3), stearidonic acid C18:4 (n-3), eicosatetraenoic acid C20:4 (n-3) and arachidonic acid C20:4 (n-6) were also observed in minor quantities.

The profile of fatty acids varied significantly ($P < 0.05$) depending on the type of process water tested. As described

Table 1 – Percentage of amino acids (AA) based on the total content of AAs from selected samples of different process waters. Results are presented as average values \pm SD ($n=3$). Essential amino acids (EAA) are denoted with an asterisk (*). Explanations to the sample codes are seen in Fig. 1. Incubation time of herring in each of the tested water is indicated between brackets.

Amino acids	RSW-S	SW-S (48 h)	PW-S	SB1-S (15 h)	SB2-S (15 h)	SB3-S (15 h)	RSW-F	SW-F (48 h)	PW-F	SB5-F (15 h)	SB4-F (15 h)
Histidine*	5 \pm 0.5	3.5 \pm 0.2	2.3 \pm 0.2	6.8 \pm 0.2	7.1 \pm 0.1	8.2 \pm 1.1	1.4 \pm 0.2	1.1 \pm 0.5	1.3 \pm 0.2	3.6 \pm 0.1	4 \pm 0.3
Isoleucine*	3 \pm 0.5	2.4 \pm 0.2	2.5 \pm 0.7	3.2 \pm 0.4	1.8 \pm 0.3	2.8 \pm 0.2	2.1 \pm 0.1	1.6 \pm 0.2	2.4 \pm 0.5	3.6 \pm 0.4	3 \pm 0.3
Leucine*	13.4 \pm 1	10.1 \pm 0.7	13.1 \pm 0.7	11.5 \pm 1.2	13.3 \pm 1.8	13.2 \pm 2	15.8 \pm 0.9	15.1 \pm 2.9	12.5 \pm 0.4	12.1 \pm 1.5	14.2 \pm 0.1
Lysine*	13.4 \pm 0.5	9.3 \pm 0.2	6.4 \pm 1.3	10.3 \pm 1.6	7.9 \pm 1.6	9.2 \pm 1.9	7.5 \pm 1.5	7.2 \pm 0.2	7.1 \pm 0.5	10.7 \pm 0.1	8.6 \pm 1.6
Phenylalanine*	5.5 \pm 1	3.9 \pm 0.1	3.9 \pm 0.4	5.6 \pm 0.3	5.7 \pm 0.1	5.7 \pm 0.5	5.1 \pm 0.1	6.3 \pm 0.7	4.5 \pm 0.7	6.7 \pm 0.4	7.5 \pm 0.1
Threonine*	2.5 \pm 0.2	3.6 \pm 0.1	4.1 \pm 0.4	3.2 \pm 0.3	3 \pm 0.2	4 \pm 0.4	3.9 \pm 0.6	3.1 \pm 0.2	3.6 \pm 0.4	3.2 \pm 0.5	2.9 \pm 0.5
Valin*	3.5 \pm 0.5	4.3 \pm 0.2	3.9 \pm 0.2	4.2 \pm 0.3	2.8 \pm 0.3	4.7 \pm 0.4	3.7 \pm 0.6	3.4 \pm 0.4	3.6 \pm 0.2	4.1 \pm 0.3	3.6 \pm 0.1
Alanine	7.9 \pm 0.5	9.7 \pm 0.8	7.3 \pm 0.2	5.2 \pm 0.1	8.7 \pm 1.7	9.1 \pm 0.7	9.5 \pm 1.5	9.7 \pm 0.2	6.7 \pm 0.2	11.4 \pm 0.8	9 \pm 1.3
Arginine	7.9 \pm 0.5	4.4 \pm 0.1	3.8 \pm 0.5	3.6 \pm 0.4	3.3 \pm 0.2	3.7 \pm 0.1	7.9 \pm 1.1	5.6 \pm 0.2	3.1 \pm 0.7	5.7 \pm 0.9	3.8 \pm 0.4
Aspartic acid + asparagine	6.9 \pm 0.9	9.2 \pm 0.9	11.6 \pm 2.3	10.2 \pm 0.3	11.1 \pm 2.2	9.5 \pm 0.8	9 \pm 1.1	11.9 \pm 0.5	10.9 \pm 1.3	7.6 \pm 1.3	9.3 \pm 1
Glutamic acid + glutamine	13.4 \pm 1.5	20.1 \pm 0.1	20.5 \pm 2.9	20.1 \pm 2.3	14.9 \pm 2.9	9.6 \pm 1.7	11.6 \pm 2.3	14.8 \pm 1.6	22.7 \pm 3.6	12.1 \pm 1.8	13 \pm 0.1
Glycine	5.8 \pm 0.5	9.5 \pm 0.1	5.8 \pm 1.3	6.8 \pm 0.3	7.5 \pm 1.2	7.3 \pm 1.2	6.8 \pm 0.5	6.8 \pm 0.5	6.5 \pm 0.6	10.5 \pm 0.9	8.5 \pm 1.2
Proline	3.4 \pm 0.4	2.9 \pm 0.2	3.6 \pm 0.9	2.7 \pm 0.3	3.9 \pm 0.3	4.1 \pm 0.3	5.1 \pm 0.4	4.9 \pm 0.5	4.4 \pm 0.6	3.6 \pm 0.3	3.8 \pm 0.3
Serine	4 \pm 0.5	5.2 \pm 0.7	7.6 \pm 1.4	4.1 \pm 0.3	5.5 \pm 0.5	6.2 \pm 0.6	7.5 \pm 0.2	8.6 \pm 0.7	7.3 \pm 0.7	2.5 \pm 0.1	4.8 \pm 0.2
Tyrosine	4.4 \pm 0.5	1.9 \pm 0.2	3.6 \pm 0.2	2.4 \pm 0.1	3.5 \pm 0.1	2.7 \pm 0.3	3.1 \pm 0.7	2.9 \pm 0.2	3.4 \pm 0.4	2.6 \pm 0.2	4 \pm 0.2
\sum EAA	46.3	37.1	36.2	44.8	41.6	47.8	39.5	34.8	35	44	43.8
\sum NEAA ^a	53.7	62.9	63.8	55.6	58.4	52.2	60.5	65.2	65	56	56.2
\sum AA	100	100	100	100	100	100	100	100	100	100	100

^a Non-essential amino acid for humans.

Table 2 – Trace elements in selected samples of process waters. Results are presented in mg L⁻¹ as average values \pm SD ($n=3$). Explanations to the sample codes are seen in Fig. 1. Incubation time of herring in each of the tested water is indicated between brackets.

Samples	Nickel	Zinc	Iron	Calcium	Magnesium
RSW-S	0.42 \pm 0.04	0.28 \pm 0.04	0.50 \pm 0.04	10.9 \pm 0.62	42.4 \pm 2.5
SW-S (48 h)	1.16 \pm 0.10	1.02 \pm 0.02	1.36 \pm 0.10	10.7 \pm 1.21	36.2 \pm 4.4
PW-S	0.16 \pm 0.02	0.46 \pm 0.05	1.70 \pm 0.20	23.1 \pm 2.20	1.95 \pm 0.4
SB1-S (15 h)	0.32 \pm 0.04	1.11 \pm 0.10	1.18 \pm 0.06	39.6 \pm 4.91	14.4 \pm 0.1
SB2-S (15 h)	0.44 \pm 0.04	1.66 \pm 0.11	0.32 \pm 0.02	22.1 \pm 1.40	20.2 \pm 2.3
SB3-S (15 h)	0.38 \pm 0.05	0.60 \pm 0.11	0.58 \pm 0.04	10.9 \pm 0.51	24.4 \pm 4.8
RSW-F	0.32 \pm 0.03	0.46 \pm 0.06	0.58 \pm 0.05	24.5 \pm 1.10	24.4 \pm 2.2
SW-F (48 h)	0.92 \pm 0.04	0.90 \pm 0.10	1.14 \pm 0.08	13.8 \pm 1.42	10.5 \pm 0.8
PW-F	0.12 \pm 0.02	0.46 \pm 0.03	1.70 \pm 0.11	23.1 \pm 0.52	2.70 \pm 0.5
SB5-F (15 h)	0.26 \pm 0.01	1.04 \pm 0.10	1.04 \pm 0.10	34.3 \pm 0.73	12.7 \pm 1.8
SB4-F (15 h)	0.46 \pm 0.03	1.28 \pm 0.14	0.68 \pm 0.10	95.7 \pm 5.41	18.1 \pm 2.1

above, the total fatty acid content was very low in RSW, and mainly MUFAs and SFAs were found; PUFA was only found in RSW-F. The major class of fatty acids observed in SW samples, at ≥ 24 h incubation, was PUFAs, followed by both SFAs and MUFAs. In contrast to SW, the major class of fatty acids observed in SB samples was MUFAs, followed by both SFAs and PUFAs. PW had a fatty acid profile similar to that obtained for SB's in that MUFAs were the major class of fatty acids; this was first followed by SFAs and then by PUFAs.

The LC $n-3$ PUFAs (EPA and DHA) were the main PUFAs found in all tested process waters. Together, EPA and DHA reached up to 93.2%, 93.8% and 87.8% of the measured PUFAs, and thereby up to 44.5%, 26.7% and 17.1% of total fatty acids in SW, SB and PW, respectively, when merging the two catching occasions and the different incubation times. It should also be stated that the incubation time and the catching occasions did not significantly affect the profile of fatty acids in the tested types of process water.

3.5. Polypeptide profile

The polypeptide profile of the selected samples described in section 2.1 is shown in Fig. 3. The protein content in RSW-S (0.33 ± 0.01 mg mL⁻¹) was significantly ($P < 0.05$) lower compared to other types of process water; thus, the amount of protein loaded in the corresponding well was lower compared to wells representing the other waters, i.e. 3.3 μ g instead of 10 μ g. Consequently, almost no bands were observed in lane 2 (Fig. 3a). Most of the polypeptides observed in RSW-F, SW-S, SW-F and PW-F were ≤ 66 kDa with an exception of a band at ~ 96 kDa in PW-S (lane 4, Fig. 3a), suggested to be α -actinin. Among the polypeptides ≤ 66 kDa, some were tentatively identified as albumin (~ 66 kDa) and actin (~ 43 – 45 kDa). Clear bands were also present at ~ 48 , ~ 29 and ~ 12 – 14 kDa. For SB samples (lanes 5–7 and 5–6 in Fig. 3a and b, respectively), polypeptides with a wider molecular weight span (~ 12 – 200 kDa) were present. Among the identified polypeptides specific to the SB's were myosin heavy chain (~ 200 kDa) and desmin (~ 55 kDa). Preliminary electrophoresis analyses on other gel types (10% NuPAGE® Bis-Tris Gels) also revealed polypeptides at ~ 600 and 2000 kDa, tentatively identified as nebulin and titin, respectively, in 5% and 8% SB's, i.e. in SB2-S and SB3-S (data not shown).

3.6. Amino acid profile

The different types of herring process water presented in Table 1 contained most of the essential and non-essential

amino acids. In RSW-S and RSW-F, the measured essential amino acids made up 46.3% and 39.5%, respectively, of the total amino acids. Corresponding values for SW-S and SW-F were 37.1% and 34.8%, respectively, while those for PW-S and PW-F were 36.2% and 35%, respectively. For SB-samples, relative amounts of essential amino acids were 41.6–47.8%, respectively. The dominant amino acids in all tested samples were leucine and glutamic acid plus glutamine. Other abundant amino acids were lysine, alanine, aspartic acid, asparagine and glycine.

3.7. Trace elements

Among the trace elements measured, only iron, zinc, nickel, calcium and magnesium were detected in the different herring process waters (Table 2). The content of nickel, zinc and iron was low in all tested samples. The highest content of nickel was found in SW samples with the lowest content found in PW samples. The highest content of zinc was found in SB samples, particularly in SB2-S and SB4-F, while the lowest content was found in RSW and PW samples. For iron, the highest content was found in PW samples, while the lowest levels were found in most SB and RSW samples. In all tested samples, calcium and magnesium were found in significantly ($P < 0.05$) higher levels than the other trace elements. The highest calcium content was found in SB4-F, followed by SB1-S and SB5-F, respectively. For magnesium, the highest content was found in RSW samples, while the lowest content was found in PW samples.

3.8. Recovery of biomolecules using electroflocculation (EF) and ultrafiltration (UF)

Three trials were conducted and the results are shown in Table 3. In trial 1, run on SB from 15 h incubation of cut D in 3% NaCl, the manual removal of the floating fat layer, observed on the surface of SB, resulted in a slight (non-significant, $P > 0.05$) decrease in the protein content and the COD value, and a significant ($P < 0.05$) decrease in the total fatty acid content. The floating fat layer contained 0.9% and 15.3% of the proteins and fatty acids found in the initial feed, respectively. The foam collected following EF treatment contained 16.6% and 29.6% of the proteins and fatty acids found, respectively, in the EF-inlet. However, following theoretical calculations, which are supported by the visual loss of foam inside the EF-equipment, corresponding numbers were 31% and 62%, respectively. After EF-treatment, the COD value thus decreased significantly ($P < 0.05$), by 30%. The subsequent use of UF resulted in a UF

Table 3 – Compositional and functional characteristics of fractions recovered in the EF/UF concentration trials applied on SB and PW samples. All data on concentrations and functional properties are shown as average values \pm SD ($n=3$). Functional properties were only tested on concentrates from trials 1 and 2; these were: foaming capacity (FC %), foaming stability (FS %) after 60 min, emulsifying activity (EAI $\text{m}^2 \text{g}^{-1}$) and emulsifying stability index (ESI %). Process waters used in the three trials were; SB produced from cut D incubated for 15 h in 3% salt (trial 1), SB produced from cut B incubated for 20 h in 5% salt (trial 2) and PW produced during the preparation of cut C (trial 3).

Stage of trial	Protein content (g L ⁻¹)	Total proteins (g)	Fatty acid content (g L ⁻¹)	Total fatty acids (g)	Dry matter (g 100 g ⁻¹)	Total dry matter (g)	COD (mg L ⁻¹)	Foaming capacity		Emulsifying capacity	
								FC (%)	FS (%)	EAI (m ² g ⁻¹)	ESI (%)
Trial 1											
Initial feed	12.6 ± 1.2	1260	3.3 ± 0.20	330	4.7 ± 0.05	4690	25.5 ± 1.8	13.0 ± 1.43	80.8 ± 3.21	19.6 ± 0.15	24.0 ± 0.04
Floated fatty layer	10.6 ± 1.1	11.6	92.8 ± 4.4	50.6	51.3 ± 1.80	300	– ^a	n.m. ^b	–	25.3 ± 1.25	27.3 ± 3.29
EF inlet	12.3 ± 1.4	1244.8	2.7 ± 0.30	271.1	4.67 ± 0.04	4614	23.1 ± 1.9	–	–	–	–
EF foam	19.1 ± 0.3	207.1	7.5 ± 0.30	80.2	8.25 ± 0.40	741	–	15.0 ± 2.06	90.1 ± 2.43	20.8 ± 1.14	24.4 ± 2.15
EF outlet/UF inlet	9.9 ± 0.1	866.8	0.9 ± 0.10	102.7	4.13 ± 0.03	3613	16.2 ± 0.5	–	–	–	–
UF permeate	2.7 ± 0.3	79.5	0.01 ± 0.001	0.27	3.22 ± 0.02	947	7.6 ± 0.09	–	–	–	–
UF concentrate	14.3 ± 0.1	744.6	1.8 ± 0.01	92.4	4.63 ± 0.02	2412	–	12.4 ± 0.74	70.5 ± 21.44	18.5 ± 0.62	17.4 ± 0.56
BSA ^c	–	–	–	–	–	–	–	89.7 ± 4.69	86.1 ± 0.35	32.1 ± 0.72	52.5 ± 3.77
Trial 2											
Initial feed	5.9 ± 0.3	590	0.17 ± 0.02	17	4.42 ± 0.01	4420	11.4 ± 0.6	49.4 ± 4.97	45.4 ± 2.89	28.2 ± 1.30	25.2 ± 1.71
UF permeate	3.8 ± 0.1	153.4	0.006 ± 0.001	0.24	4.78 ± 0.02	1926	3.80 ± 0.08	–	–	–	–
UF concentrate	8.1 ± 0.1	481.3	0.27 ± 0.01	16.2	2.82 ± 0.03	1676	–	66.0 ± 10.96	93.3 ± 0.94	23.8 ± 0.01	39.8 ± 1.73
BSA ^c	–	–	–	–	–	–	–	76.7 ± 5.84	81.1 ± 1.83	31.4 ± 0.59	48.2 ± 3.17
Trial 3											
Initial feed	0.81 ± 0.02	81	0.047 ± 0.02	4.7	0.89 ± 0.02	890	1.80 ± 0.06	–	–	–	–
UF permeate	0.64 ± 0.02	48	0.007 ± 0.001	0.53	0.97 ± 0.01	728	1.05 ± 0.06	–	–	–	–
UF concentrate	1.18 ± 0.06	29.5	0.145 ± 0.01	3.63	0.55 ± 0.02	138	–	–	–	–	–

^a Not analyzed.

^b Not measurable.

^c Bovine serum albumin (BSA) was used as standard protein in functionality testing.

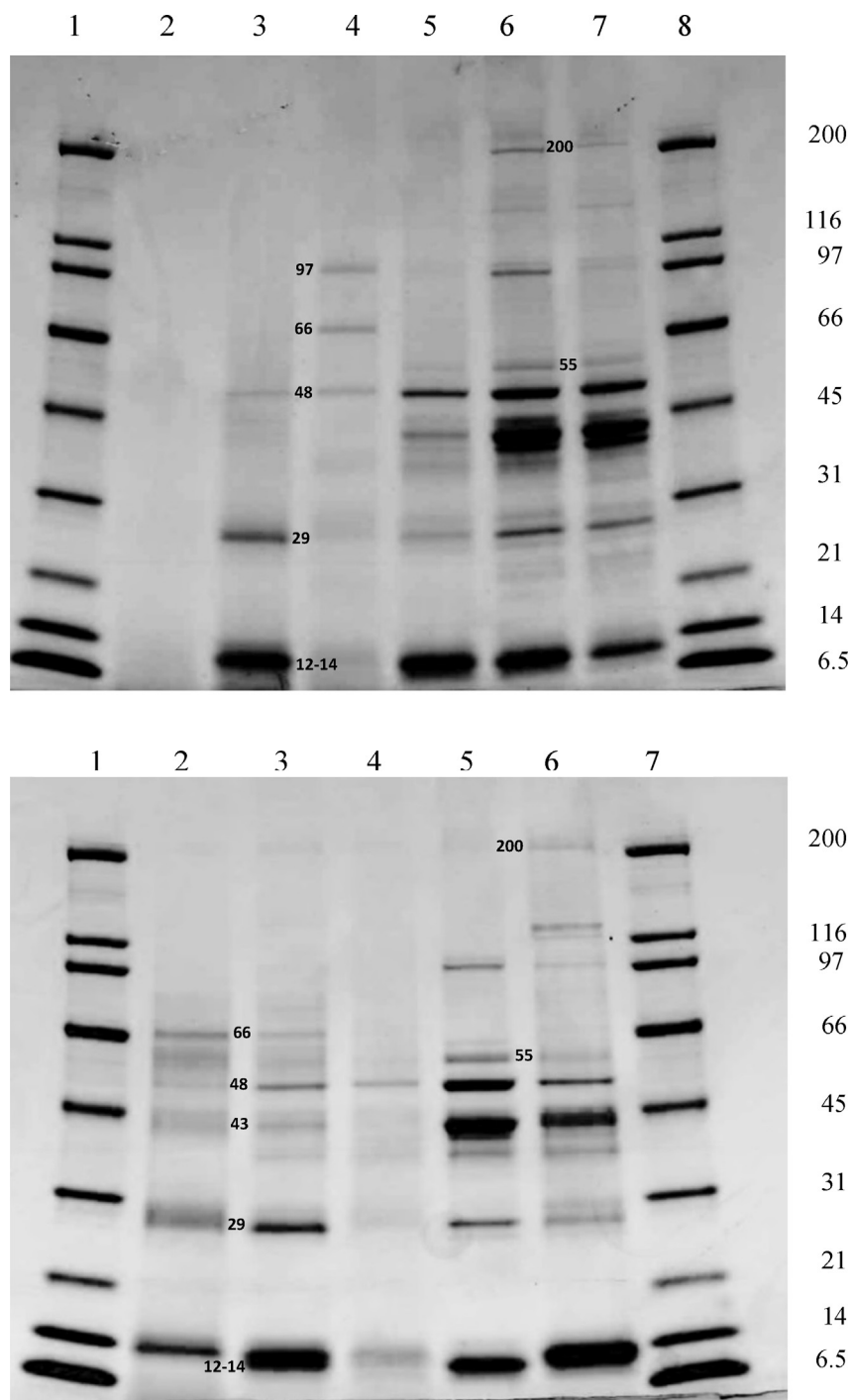


Fig. 3 – Polypeptide profiles of selected samples from the different process waters in September (A) and February (B), analyzed by SDS–PAGE. The lanes in A represent (1) the protein marker, (2) RSW-S, (3) SW-S: (48 h incubation), (4) PW-S, (5) SB1-S (15 h incubation), (6) SB2-S (15 h incubation) and (7) SB3-S (15 h incubation), respectively. The lanes in B represent (1) the protein marker, (2) RSW-F, (3) SW-F (48 h incubation), (4) PW-F, (5) SB5-F (15 h incubation) and (6) SB4-F (15 h incubation). Ten μg proteins were loaded in each lane, except in lane 2 of panel A, where 3.3 μg was loaded. Explanations to the sample codes are seen in Fig. 1.

permeate with a significantly ($P < 0.05$) lower concentration of proteins and fatty acids compared to the UF inlet, while the UF concentrate contained significantly ($P < 0.05$) higher amount of proteins and fatty acids compared to the UF inlet (Table 3). As a result, the UF process decreased the COD value significantly ($P < 0.05$) by 53%, and recovered 85.9 and 89.9% of the proteins and fatty acids found, respectively, in the UF inlet. The concentration factor in this trial was 1.6.

In trial 2, run on SB from 20 h incubation of cut B in 5% NaCl, the application of UF without prior EF treatment resulted in a

UF permeate with a significantly ($P < 0.05$) lower concentration of proteins and fatty acids, compared to the initial UF inlet (Table 3). This was concomitant with a significant ($P < 0.05$) increase in the protein and fatty acid concentration in the UF concentrate. The UF process decreased the COD value significantly ($P < 0.05$) by 66.7%, and recovered 81.6% and 95.3% of the proteins and fatty acids found, respectively, in the UF inlet. The concentration factor in this trial was 1.9.

In trial 3, run on PW produced during the preparation of cut C, the application of UF without prior EF treatment resulted in

a UF permeate with a significantly ($P < 0.05$) lower concentration of proteins and fatty acids, compared to the initial UF inlet (Table 3). This was also simultaneous with a significant ($P < 0.05$) increase in the protein and fatty acid concentration in the UF concentrate. The UF process recovered 36.4% and 77.1% of the proteins and fatty acids found, respectively, in the UF inlet and decreased the COD value significantly ($P < 0.05$) by 41.7%. The concentration factor in this trial was 4.

The mass balance for total proteins and fatty acids was followed for all the concentration trials and was considered satisfactory. In trial 1, 82.8% and 70.8% of the initial proteins and fatty acids, respectively, were found in the different recovered fractions and in trial 2, 107.6% and 96.7% respectively, were found. Likewise in trial 3, 95.6% and 88.5% of the initial proteins and fatty acids, respectively, were recovered. There was a marginal loss of protein and fatty acid mass in the tubing of the UF system, while it is evident, from Table 3, that there was a more observable loss in protein and, especially, in fatty acid mass after the use of EF. In fact, a thick and sticky layer of fat was observed on the inner walls of the EF cell. This layer was not included in the data shown in Table 3, but may contributed to the observed loss in the proteins and fatty acid mass in trial 1.

3.9. Functionality of recovered concentrates

Foaming (FC, FS%) and emulsifying properties (EAI $\text{m}^2 \text{g}^{-1}$, ESI %) of initial feeds and concentrates recovered in trials 1 and 2 are shown in Table 3. Generally, FC was significantly lower ($P < 0.05$) for samples from trial 1 than trial 2, which was not the case for FS%. In trial 1, FC was only 13.7–16.7% of that for the reference protein BSA, while in trial 2, it was 64.4–86.0%. Within trial 1, the application of EF and UF did not significantly alter FC as compared to the initial feed; however, the manually floated fat-rich layer did not foam at all. Further, FS% was significantly higher ($P < 0.05$) for the EC foam than UF concentrate; the former even exceeding the FS% for the reference protein. In trial 2; both FC and FS% were higher after UF concentration with FS% for the UF concentrate exceeding that for the reference protein.

Regarding emulsification, EAI and ESI were in the same range for samples from trials 1 and 2; also when comparing to the respective analyses of the reference protein. Within trial 1, EAI and ESI were significantly higher ($P < 0.05$) for the manually floated layer than for the other samples. EF and UF per se did not significantly affect EAI, while a significant reduction ($P < 0.05$) in ESI was seen after UF concentration. In trial 2, EAI and ESI significantly decreased and increased ($P < 0.05$), respectively, after UF concentration.

Looking at the data for the reference protein, it seemed that the conditions of trial 1, e.g. with a somewhat lower IS than in trial 2 (1.79% vs. 3.24% NaCl), were slightly more favorable for retaining foaming and emulsification properties of the biomolecules.

4. Discussion

Substantial volumes of different types of process water are produced during the production of marinated herring; some of which according to this study have the characteristic of containing high amounts of organic matter, mainly in the form of proteins and lipids. To date, no previous work has looked comprehensively into the composition of the different types of process water generated in the early steps of producing

marinated herring. Besides, no previous technological solutions have been described in the context of concentrating the organic matter found in these process waters.

4.1. Composition of herring process waters

The dry matter content of the studied herring process waters was more influenced by their salt content, than by their organic matter content. The dry matter content in PW samples, which had higher organic matter content than RSW samples, was the lowest, since cutting was the only step comprising fresh water. In the different salt containing water types, the initial amounts of salt (i.e. 3%, 5% and 8%) were clearly reflected in the dry matter estimations, but there were still significant deviations not only due to leaching of the organic matter from the herring but also due to the continuous exchange of salt and water taking place between the fish and the surrounding solution during storage. It is known that there is an osmotic pressure driven flux of salt into the tissue and a flux of water out of the tissue when fish is placed in a solution with salt levels above the physiological levels (i.e., $\geq 0.9\%$). The contribution of the initial salt level to the dry matter of the process waters thus decreases during the incubation until an equilibrium salt level is reached inside and outside the fish (Andersen et al., 2007). Limited analyses of ash content in SW-S (data not shown) confirmed how the ash level of the water decreased over time; in this case, from 3.2% to 1.5% after 96 h incubation.

The observed leakage of proteins from herring tissues into the different types of process waters was affected by the incubation time and the herring cut. Regarding incubation time, the highest protein content in SW and SB samples was usually obtained at the end of incubation. Similarly and despite the fact that herring was intact in both RSW and SW, the protein content in SW samples was higher than that in RSW due to the longer incubation time of herring in SW (in total 96 h as opposed to 5 and 20 h in RSW-S and RSW-F, respectively). Increased muscle exposure most likely explained why cut D (SB4-F) released more protein into a 3% salt solution than cut A (SB5-F) and whole herring (SW) (e.g. Fig. 2b). Higher exposure of muscle probably also explained why PW samples had higher protein levels than RSW, despite the former being exposed to the fish for a shorter time period, and despite being deficient in salt. It is well known that salt increases the solubilization of myofibrillar proteins up to a level of around 1 M (i.e. 5.8% NaCl) (Stefansson and Hultin, 1994), after which a salting-out effect (precipitation of proteins) usually appears. In the presented data set, there were no samples that could be directly compared to visualize the known effect of salt on protein solubilization, since also the cuts differed when salt levels of process waters differed (Fig. 1). It is, however, important to stress that it is a confounding effect to both herring incubation time and cut.

Since very little is done in the field of herring process waters, it is hard to find compositional data comparable to the ones presented here. Nevertheless, Christensen et al. (2011) found that the blood brine formed 2 days after traditional dry salting of herring (1 part salt: 9 parts fish) was determined to contain 1% protein, 18.8% dry matter and 17% salt. Andersen et al. (2007) found that the protein content in similar salt brines increased from 1% to 4% in 45 days storage of old fashion salting of herring. In herring marinating brines, i.e. with salt and acetic acid present, both incubation time and increased degree

of herring muscle exposure increased total nitrogen content in residual marinades (Szymczak and Kołakowski, 2012)

Although not very visible in terms of total protein levels, SDS-PAGE data revealed that higher salt brines (5% and 8%) (lanes 6 and 7, respectively in Fig. 3a) solubilized larger amounts of myofibrillar proteins like titin, nebulin (not showed), myosin heavy chain and desmin compared to SB's obtained from 3% salt solutions (lane 5, Fig. 3a and lanes 5–6 in Fig. 3b). Further, the release of high molecular weight myofibrillar proteins was triggered by increased muscle exposure, explaining why polypeptides >66 kDa were slightly more abundant in the SB sample produced from the incubation of cut D in 3% salt solutions (lane 6 in Fig. 3b) compared to other more intact samples incubated at the same salt concentration (lanes 2, 3, 5 in Fig. 3A and lanes 2, 3 in Fig. 3B).

All essential amino acids were found in the different process waters, which were characterized by having high relative contents of leucine, lysine, alanine, aspartic acid and asparagine, glutamic acid and glutamine and glycine. This correlates with earlier studies showing that herring muscle is high in these amino acids (Iwasaki and Harada, 1985; Usydus et al., 2009).

The fatty acid content in the process waters was mainly affected by the herring cut, and was the highest in SB samples followed by PW, SW and finally RSW samples. Further, among the SB samples, those produced from cut C and cut D (SB3-S and SB4-F, respectively) had higher total fatty acid content compared to cut B (SB2-S) and cut A (SB1-S, SB5-F). Long incubation times led to high total fatty acid content in SW samples, while incubation time had no clear effect on the total fatty acid content in SB samples, very likely due to the difficulties associated with taking representative SB samples with respect to fat. Indeed, a fatty layer always floated at the surface in SB, and due to the large volumes of the containers (1000 L), it was very difficult to obtain representative samples. This, however, did not seem to affect protein analysis in SB samples, and the sampling problem was not evident for any of the other process waters. It is known that herring in extreme cases can vary from a few percentages up to 30% in fat (Ekstrand et al., 1993), with minimum levels being found in the end of the spawning season (Kołakowska et al., 2002). Although 5 months passed between the two samplings, no clear seasonal effects on total fatty acid contents were obtained, beside perhaps a small tendency for higher levels in SB-S than SB-F samples. This was in line with the surprisingly small differences in the fat content of the two catches, which was estimated by the producers to be around 12% for both catches. It is however important to be aware that herring process waters might vary quite extensively in fat, but the exact degree of such variations could not be revealed and should be the subject for a separate study.

In terms of the fatty acid profile, all tested types of process waters had the typical profile for herring, as the major fatty acids observed were MUFAs, especially 20:1 and 22:1 (Jensen et al., 2007; Lindqvist et al., 2009). Nonetheless, SW samples clearly deviated from the above typical profile, as the major fatty acids found were PUFAs. The most likely reason could be the fact that SW was rich in blood, and was therefore influenced by the blood lipid profile; both plasma and erythrocyte membranes are rich in PUFAs (Rise et al., 2007). The most valuable fatty acids are the LC *n*-3 PUFA, and from this perspective, some of the studied types of process waters, such as SB's and SW, could be interesting novel sources of LC *n*-3 PUFA. In a few years, it is estimated that the current fish oil production will not cover the demand for LC *n*-3 PUFA within the feed, food and

nutraceutical industries; therefore, novel sources are urgently needed (Jacobsen et al., 2013).

It was also clear that minerals leaked out to waters used during herring processing. The significantly higher content of calcium and magnesium compared to the rest of the minerals was in line with earlier studies on herring tissue (Torelm and Danielsson, 1998) and is most likely due to the leaching of these two trace elements from bones into the RSW, SW, PW and SB samples. It should be stressed that de-boning of herring allows small bones to follow into the pre-salting stage. In the analyzed herring process waters, iron was generally more abundant than zinc, especially in RSW, SW and PW samples. This was most likely due to the leakage of blood into these waters. It has earlier been reported that zinc is more abundant in herring compared to iron (Torelm and Danielsson, 1998).

Based on the above, it is evident that a substantial loss of interesting biomolecules into the process waters takes place during the early steps of marinated herring production. Calculations based on our data showed e.g. that ~9.2 and ~4.1 kg of pure proteins and fatty acids, respectively, are lost per ton of processed herring. Estimating an average protein and fat content in the herring raw material of 18% and 10%, respectively, the proteins and fatty acids lost in process waters constitutes ~20% and ~15% of the total herring proteins and fatty acids, respectively. Thus, it is essential to develop an environmentally friendly process for the concentration of these biomolecules for their utilization and commercialization.

4.2. Concentrating herring biomolecules with EF and UF

The applied concentration process for proteins and lipids was based on using EF as a pre-treatment step and UF using SiC membrane as the main concentration technique. EF has already been described for separating organic molecules without the addition of any chemicals (Barrera-Díaz et al., 2011). The use of EF in trial 1 was promising due to the significant recovery of proteins and fatty acids in the tested SB, and the relatively short time in which the pre-treatment was accomplished, ~1 h for 100 L. Nevertheless, the use of EF has earlier proved to remove significantly more organic matter from other types of process water, compared to what was reported in this study. For instance, Valero et al. (2011) reported the removal of 75–80% of total organic carbon, 75% of total nitrogen and 99% of total suspended solids from the wastewater of almond industry. Also, Tsai et al. (2002) demonstrated that EF removed 82% of total suspended solids and 14% of soluble organic matter found in poultry processing wastewater. However, it should be kept in mind that the pilot EF unit used in this work was custom made, with limited control over the process. Therefore, it is very likely that more proteins and fatty acids can be recovered from herring process waters once all operational parameters are optimized. For the UF experiments conducted in this work, more than 80% of both proteins and fatty acids were recovered when a 5% SB from cut B was used (trial 2). In trial 3, however, much lower amounts of proteins and fatty acids were recovered; 36 and 77%, respectively. One main reason can be that this trial was carried out on PW, which according to the SDS-PAGE analyses contained lower molecular weight proteins than those found in SB; possibly permeating more easily through the membrane. This indicated that membranes with a pore size smaller than 0.04 μm might be required to efficiently recover proteins from PW. The relatively high recovery of fatty acids in all the trials is

expected to be the outcome of the very hydrophilic nature of SiC, which hindered the permeation of lipids through the membrane. One drawback of the applied process, however, was the lack of temperature control, which was due to the design of the pilot unit. Another drawback was the long operation times required to obtain a good concentration factor. This was mainly due to the decrease in the flux and permeability of the membrane, as the tested process waters contained high amounts of proteins and lipids, especially in trial 1 and 2. The fat most likely formed a layer on the surface of the membrane, due to the applied pressure, which slowed down the recovery process.

The application of UF for treating wastewater from the seafood industry has been reported by several other researchers and proved to be a promising technique to improve the competitiveness of the production processes in this industry segment. For example, up to 96% of the proteins could be recovered from shrimp shell wastewater using zirconium dioxide membranes (Benhabiles et al., 2013) and 77% of the proteins, found in sardine press liquor, were recovered using alumina and zirconite membranes (Gálveza et al., 2011). Furthermore, Afonso et al. (2004) reported that up to 62% proteins could be recovered from fish meal effluents using UF, while the combination of micro- and ultrafiltration recovered 69% of the proteins found in these effluents. In the present study, the recovery of proteins and lipids was very satisfactory (>80%), but further improvement in the process can be achieved if optimization trials are conducted to improve the process robustness in terms of recovery, flux and operation time. The latter would also allow for fair estimations of the cost-effectiveness of EF/UF for protein and fatty acid recovery from herring process waters. It should be stressed though that this work aimed to test the concept of recovering the biomolecules from herring industry process waters, rather than optimizing the recovery processes.

Regarding the technical functionality of recovered material, an important feature in potential food/feed applications, the brief testing applied here revealed that both EF and UF in most cases either did not affect, or improved, foaming and emulsification properties of biomolecules recovered in concentrates. For example, large improvements were seen in FC, FS% and ESI when subjecting SB from 20 h incubation of cut B in 5% NaCl to UF (trial 2). A major compositional change during UF is the removal of low molecular weight (LMW) compounds as peptides and free amino acids. It is known that LMW-peptides cannot form a cohesive interfacial film (Damodaran, 2008). Further, the temperature raise during trial 2 (from 10–12 °C to ~20 °C) most likely induced partial protein denaturation; which is known to improve foaming and emulsifying properties (Damodaran, 2008). The significantly better ability of samples from trial 2 to create foams as compared to samples from trial 1 could be due to the lower fatty acid content in samples from trial 2 (Table 3); lipids are more surface active than proteins so they inhibit the absorption of proteins at the air-water interface (Damodaran, 2008). This shows that endogenous features of herring process waters can have larger effects on functionality than EF or UF per se.

The presented recovery and functionality of proteins and lipids from herring process waters, along with the simultaneous significant decrease of the COD values, and the earlier reports on successful UF-treatments of other types of seafood process waters (Afonso et al., 2004; Benhabiles et al., 2013; Gálveza et al., 2011) suggest that EF and/or UF can represent interesting ways to avoid that marine biomolecules with a

high nutritional value end up as effluents, which currently prevent their potential upgrading to food or feed ingredients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fbp.2015.08.002>.

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PAPER I

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Research Article

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Chemical Characterization, Antioxidant and Enzymatic Activity of Brines from Scandinavian Marinated Herring Products

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Abstract

Brines generated during the last marination step in the production of marinated herring (*Clupea harengus*) were chemically characterized and analyzed for antioxidant and enzyme activities. The end-products were vinegar cured, spice cured and traditional barrel-salted herring with either salt or spices. The chemical characterization encompassed pH, dry matter, ash, salt, fatty acids, protein, polypeptide pattern, iron and nitrogen. The antioxidant activity was tested with three assays measuring: iron chelation, reducing power and radical scavenging activity. The enzymatic activity for peroxidase and protease were also tested. Results revealed that the brine can contain up to 56.7 mg protein/mL, up to 20.1 mg fatty acid/mL, good antioxidant activity, high amounts of the antioxidative amino acids lysine, alanine, and glycine, and high enzymatic activity. The potential of using the protein-rich fraction with biological activity from brines from the marinated herring production was demonstrated in this work.

Keywords: Herring (*Clupea harengus*); Brine; Wastewater; Antioxidant (iron chelating, reducing power and ABTS radical scavenging); Enzymatic (peroxidase and protease)

Introduction

The food sector produces large volumes of waste, both solids and liquids, resulting from the production, preparation, and consumption of food and this represent an important loss of valuable biomass and nutrients. In addition, with the growing population and the concerns about environmental pollution, today's society sets focus on our limited resources and on optimizing the utilization of the available raw materials. Consequently, there is a considerable emphasis on the recovery, recycling and upgrading of our organic waste in order to extract as much value as possible from them [1-3]. Therefore, it is important to also propose new solutions to food producers to optimize existing processes in order to both reduce the amount of waste generated and extract more value from the residual raw material that is wasted.

In the fish industry, up to 50% of the raw material is commonly discarded [4], and there is an increasing interest within the seafood sector to explore new possibilities for their utilization [5,6]. In Norway, almost 800 000 tons of by-products were generated in 2009, of which approximately 77% were exploited and the remaining 23% were dumped. More than 80% of the exploited by-products were used for the production of low value-added products such as fish meal, silage and feed whereas only about 15% were used for human consumption [7]. Research has shown that marine by-products contain compounds such as minerals, fatty acids, amino acids, polysaccharides and proteins with interesting biological activity [8-11]. Sathivel et al. [12] demonstrated that protein hydrolysates made from herring by-products (head and gonad) may serve as a good source of desirable quality peptides and amino acids.

Besides the solid by-products, a large amount of liquid waste is produced in the fish industry. A well-known example is the wash water from surimi production. Stine et al. [13] showed that proteins in such wastewater could be recovered and added back to the surimi without affecting the end-product quality. Another type of product which, during production, generates large amount of liquid waste is the marinated herring, a traditional Scandinavian product. The liquid waste generated from the production of these marinated products

can in total reach a volume of more than 700 L per 100 kg herring produced; of which ~200 L come from the maturation step and onwards. Since the European Union annually lands almost 960 000 tons of herrings, of which a large proportion ends up as marinated product [14], there is a huge amount of liquid by-products available for value adding from this production in Europe. A crucial part of this type of production is the maturation step in which fillets or whole fish can be stored in salt brine from a few months and up to two years. During this period, many biochemical reactions takes place liberating amino acids, peptides, enzymes and lipids into the brine [10]. The brine from marinated herring has been characterized in order to better understand the ripening process of barrel-salted herring [8,9,15,16]. Christensen et al. [17] tried to re-use such brine in a fresh batch of traditional barrel-salted herring in order to speed up the maturation time, but this study showed no effect of the added brine on the processing time. Apart from these studies, a thorough characterization of brines containing spices and desalting brines has, to the best of our knowledge, never been performed. Furthermore, investigation of the potential utilization of herring marinating brines has not been previously reported.

In this work, we aimed to characterize brines from the last steps in the production of marinated herring (i.e. from the maturation step and onwards) in order to identify compounds with any potential commercial value that can be exploited from this by-product streams. In addition to the basic chemical characterization, antioxidant activity and enzymatic activity, i.e. peroxidase and protease, were also investigated. Four types of marinated end-products were considered; vinegar cured, spice cured and two traditional barrel-salted products; one with salt and one with spices.

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Materials and Methods

All chemicals and reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Steinheim, Germany). See-Blue pre-stained standard was purchased from Life Technologies (Nærum, Denmark) and Coomassie brilliant blue from AppliChem (Darmstadt, Germany).

Herring brine production

Brines from the marination steps in the production of marinated herring (*Clupea harengus*) were supplied from Lykkeberg A/S (Hørve, Denmark), and were all prepared according to Lykkeberg A/S production protocols from herring caught in the North Atlantic. Six brines were obtained from the production of four different types of marinated products, as shown in Table 1. Four brines were original brines; i.e. the brines from vinegar cured fillets (VC), spice-cured fillets (SC), traditional salted whole herring (TSa) and traditional salted spice-cured whole herring (TSp). The two remaining brines were desalting brines from TSa and TSp (D-TSa, D-TSp). TSa and TSp results from salting whole herring, filleting and then placing the fillets back in the brine for additional ripening, whereas SC and VC are produced as fillets or bites. Brine samples were divided into appropriate aliquots, transported on ice to the laboratory, stored at -80°C and thawed on ice water prior to experiments.

Proximate composition

The pH was measured directly in the brine using a Metrohm 827 pH meter (Switzerland). Dry matter (DM) content of the different brines was measured by a two-step evaporation of water at 60°C for 24 h followed by 105°C for 24 h. The %-DM was calculated as (mass of dry sample * 100) / (mass of wet sample). Ash content was measured by burning at 600°C for 24 h, and is reported as (mass of ash * 100) / (mass of wet sample). The salt content was measured by potentiometric titration of chlorine ions using AgNO₃ according to the AOAC standard method [18]. The iron content was measured by complete digestion of the sample and subsequent analysis by inductively coupled plasma mass spectrometry, ICP-MS (Perkin-Elmer SCIEX, ELAN 6000). For sample digestion, 1 g of brine was mixed with 5 ml HNO₃ (67-69%), 3 ml H₂O₂ (30%) and 0.5 ml HCl (37%), and digested for 10 min in a microwave (Multiwave 3000, Anton Paar, Austria) at 1400 W, according to the Application Notes from Anton Paar. Prior to ICP-MS analysis dH₂O was added to a sample volume of 10 ml. The iron content was calculated from a standard curve made with Titrisol® iron standard (Merck, Darmstadt, Germany).

The fatty acid analysis of the different brines was completed by solvent extraction followed by methylation and detection by gas chromatography mass spectrometry, GC-MS, of the different fatty acids [19,20]. In short, 20 mL ice-cold chloroform: methanol (1:2, containing 0.04% butylated hydroxytoluene), 40 µL standard (C:17,

2 mg/mL, dissolved in toluene) and 2 mL of raw brine sample was vortexed for 20 sec. Subsequently 8 mL of NaCl (0.05%) was added and vortexed for 30sec. and centrifuged (Heraeus Multifuge 1 S-R, Thermo scientific, Sweden) for 6 min at 2 000g, 4°C. The chloroform phase was collected and left to evaporate under nitrogen gas and re-dissolved in 2 mL toluene. Then, 2 mL of 10% (v/v) acetyl chloride in methanol were added and vortexed for 20 sec. The tubes were placed in a water bath (70°C) for 2 h and then cooled under cold running water. One mL of dH₂O and 2 mL of petroleum ether were added and vortexed for 20 sec and then centrifuged at 1000 g for 5 min. The upper organic phase, i.e. petroleum ether, was collected and evaporated under nitrogen at 40°C. Evaporated samples were then dissolved in 1 mL isooctane, from which 200 µL were used for GC-MS analysis.

Methylated fatty acids were analyzed by GC-MS using Agilent Technologies 7890A GC system connected to Agilent Technologies 5975C inert XL E1/C1 MSD with triple axis detector (Kista, Sweden). One µL of the methylated sample was injected into a split/splitless capillary injector (275°C), with a split ratio of 15:1. Fatty acid separation was conducted on a DB-WAX column from Agilent technologies (30 m×250 µm×0.25 µm) using helium as carrier gas at a flow rate of 1 ml/min. The oven was initially programmed at 100°C, constant increase of 4°C/min to 250°C, kept constant at 250°C for 4 minutes; total run time was 41.5 min. During the analysis, the MS temperature was 230°C, the MS quadrupole was 150°C and the electron energy was 70 eV; all data were acquired in scan mode with an m/z range of 35 – 500. The GC chromatograms and the MS spectra were analyzed by MSD chemstation G1791EA software provided by Agilent Technologies. The different fatty acids were identified by comparing with fatty acid standards. The standard C:17 was used to estimate the amount of each fatty acid (mg/ml) in the samples. Total fatty acid content was calculated as the sum of all detected fatty acids.

The nitrogen content was determined by Kjeldahl method, according to the AOAC standard method [21]. The protein content (mg/mL) of the brines was obtained using the BCA kit (Thermo Scientific, Pierce®, Rockford, USA) with Bovine Serum Albumin (BSA) as standard. This protein determination assay was tested for its salt stability using a 16% salt solution (highest level found in the brines) and was not found to be affected by salt. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to separate the different protein/polypeptides according to their molecular weight. The brines were diluted to a protein concentration of 1 mg/mL and mixed with Laemmli sample buffer (1:1) with 10% DDT (v/v), boiled for 3 min and centrifuged for 3 min at 13 684 g (Heraeus® Biofuge® Pico, Kendro, UK). Fifteen µL of the sample (7.5 µg protein) were loaded onto the wells of 10% NuPAGE® Bis-Tris Gels (Life Technologies™, Denmark) and run with MOPS 3-(N-morpholino)propanesulfonic acid running buffer at 200 V for 50 min. See-Blue was used as standard (10 µL was loaded on the gel). Gels were stained with Coomassie Brilliant blue G-250 overnight and washed with a de-staining solution (15% ethanol,

Product	Brine	Salt	Sugar	Acetic acid	Spice-mix	Processing time (days)
Vinegar cured (fillet)	VC	yes	no	yes	No	min 35
Spice-cured (fillet)	SC	yes	yes	no	Yes	min 42
Traditional salted (fish [†])	TSa	yes	no	no	No	180-550
Traditional salted spice-cured (fish [†])	TSp	yes	yes	no	yes	180-550
Desalting brine from TSa (fillet)	D-TSa	only fresh water				1
Desalting brine from TSp (fillet)	D-TSp					1

[†] whole fish for 6 month, then fillet and placed back in the brine

Table 1: General product information of the six brines analyzed. The processing time for the specific samples taken for this study were ~200 days for VC and SC, and ~450 days for TSa and TSp, respectively.

5% acetic acid) until protein bands became clearly visible in a colorless gel matrix.

Free and total amino acids

Free amino acids and total amino acids were analyzed, according to the method described by Farvin et al. [22]. For free amino acids determination, the amino acids were derivatized using the EZ: Fast kit from Phenomenex A/S (Allerød, Denmark). Two μL of the sample were injected into the HPLC fitted with the reversed phase column EZ: Fast AAA-MS (250 x 3.0 mm, Phenomenex A/S, Allerød, Denmark), and eluted at 35°C with a flow rate of 0.5 mL/min. The mobile phase A (water) and B (methanol) both contained 10 mM ammonium formate. The gradient consisted of linear increase from 60 to 83% B in 20 min, and then the column was re-equilibrated to 60% B until the end of the run (26 min). The eluate was transferred to the on-line MS (Agilent 1100, Agilent Technology, Waldbronn, Germany) where amino acids were ionized using APCI with scanning from 100 to 600 m/z. The amino acids were quantified based on peak areas of internal standards. For total amino acids, a sample of 500 μL brine was hydrolyzed for 1h in a microwave (same as for iron), at 110°C at 500 W, in 500 μL of 12 M HCL in sealed ampules. The samples were diluted in NaCO_3 and filtered through a 0.2 μm membrane filter before the derivatization and the analysis of amino acids content by LC-MS, as described for the free amino acids. It should be notified that this procedure did not allow the detection of methionine, tryptophan and cysteine. For both experiments, amino acid content is expressed in g/L brine.

Antioxidant assays

All the brines were analyzed using three assays for antioxidant activities; metal chelation, reducing power and ABTS-radical scavenging. To test the concentration dependency of the antioxidant activities, all the brines were analyzed in the crude, undiluted version and following sequential threefold dilutions, until a steady level was observed. They were measured on a spectrophotometer (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instruments, Inc., Vermont, USA), and tested with high salt solution (16%) prior to analysis and none of them were affected by this salt concentration.

Iron (II) chelating activity of the different brines was assayed by the method described by Farvin et al. [23] adjusted to microplate detection. Brines subjected to the different dilutions were centrifuged (13 684 g, 3 min, Biofuge® Pico, Kendro, UK) and 100 μL of the supernatant was mixed with 135 μL dH_2O in the microplate. The reaction was initiated by 5 μL of 2 mM ferrous chloride, and inhibited by 10 μL of 5 mM ferrozine after 3 min reaction time. After 10 minutes, the absorbance was measured at 562 nm. An assay control (dH_2O instead of the sample) and a sample control (dH_2O instead of reagents) were included. EDTA (200 μM) was used as a positive control and was subjected to the same dilutions as the samples. The iron chelating activity (%) was calculated as:

$$\text{Fe}^{2+} \text{ chelating activity}(\%) = \frac{\text{blank} - (\text{sample} - \text{sample control})}{\text{blank}} \times 100$$

The reducing power was measured according to the protocol described by Oyaizu et al. [24]. After centrifugation, as described above, 200 μL of supernatant was mixed with 200 μL of 0.2 M phosphate buffer (pH 6.6) and 200 μL of 1% potassium ferricyanide. The mixture was incubated for 20 minutes at 50°C. Subsequently, 200 μL of 10% TCA was added and an aliquot of 100 μL was mixed with 100 μL dH_2O and 20 μL of 0.1% ferric chloride in the microplate, and incubated 10 min at room temperature. The absorbance was measured at 700 nm.

Controls consisted of dH_2O instead of sample and reagents. Ascorbic acid (500 μM) was used as a positive control and was subjected to the same dilutions as the samples. Results are expressed as OD_{700} for the different samples at the different dilutions.

The radical scavenging activity was determined according to the method described by Re et al. [24,25] with some modifications. In short, an ABTS reagent (1:1 (v/v) consisting of 10 mM ABTS and 5 mM potassium persulfate, both in dH_2O) was left in the dark over night at room temperature. ABTS working solution was prepared by mixing ABTS reagent with borax buffer (0.1 M, pH 9). Fifty μL of sample were mixed with 200 μL ABTS working solution in the microplate and read after 1 minute at room temperature at 734 nm. Trolox (2.5 mM, in 96% ethanol) was used as a positive control and was subjected to the same dilutions as the samples. Controls were included consisting of dH_2O instead of the reagents and pure dH_2O and 96% ethanol. Results are expressed as OD_{734} for the different samples at the different dilutions.

Enzymatic activity

The brines were analyzed for two types of enzymatic activity; peroxidase and protease activity. Both methods were tested for stability with high salt solution (16%). The peroxidase activity was not affected by this high salt concentration but the protease activity was. Thus this analysis was completed on brines that had been desalted, with PD-10 desalting columns, according to instructions from the kit manufacturer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using dH_2O as an equilibration medium, prior to the assay. Both analyses were conducted in triplicate.

The peroxidase activity was measured by a semi-quantitative analysis, according to Osman et al. [26] with some modifications. In short, 50 μL brine (centrifuged at 13 684 g for 3 minutes) and 850 μL phosphate buffer (0.5 mM, pH 6.6) were mixed and used as blank. Fifty μL quercetin (1 mM in dimethylformamide (DMF)) and 50 μL H_2O_2 (20 mM) were added and the absorbance at 370 nm was measured immediately (Shimadzu UV-1800 Spectrophotometer, Holm&Halby, Denmark). Results were expressed as $\Delta\text{Abs}/\text{min}/\text{mL}$.

The protease (caseinolytic) activity was measured on the desalted brines using a green fluorescence [27] EnzChek Protease kit (Life Technologies, Denmark). In short, 100 μL brine (centrifuged at 13 684 g, 3 minutes) were mixed with 100 μL working solution (10 mM Tris-HCL, pH 7.75, 25°C) and the absorbance was measured at excitation/emission of 485/530 nm at one minute interval during an hour at 25°C (Spectra Max Gemini, Molecular Devices, UK). Results were expressed as $\Delta\text{RFU}/\text{min}/\text{mL}$ (RFU being Relative Fluorescence Units).

Statistical analysis

The measurements were carried out in triplicate on two independent aliquots of brine unless otherwise stated. The results are given as mean values \pm absolute standard deviations for independent aliquots. For all statistical analysis, the GraphPad Prism® software Ver. 4.03 was used with $p < 0.05$. Results were compared using one-way ANOVA test with Turkey's posttest or two-way ANOVA test with Bonferroni posttest.

Results and Discussion

Proximate composition analysis

The six different brines, VC, SC, TSa, D-TSa, TSp and D-TSp, obtained from the production of four different end-products, see Table 1, were characterized for their basic biochemical composition. Table 2 summarizes the pH, dry matter (DM), ash, salt, iron and total fatty acid

(TFA) content in the brines. Except for VC, the pH ranged from 5.72 ± 0.01 to 6.96 ± 0.05 in all the brines. These values are in accordance with the values found by Nielsen [10], who studied the ripening of barrel-salted herring during 189 days in which the pH-values were reported to be around 6. The pH in VC was significantly ($p < 0.05$) lower, namely 4.03 ± 0.00 , which is in accordance with earlier values found in different vinegar brines from four different brands; 3.85 - 4.35 [28].

The DM and ash content of the six brines were very different. TSa and TSp contained 37.88 ± 0.34 and 26.62 ± 0.09 wt% DM, respectively. However, the ash content in these two brines was identical indicating a much higher percentage of organic material in TSa compared to TSp. This cannot be explained by the differences in curing time of the herring (both ca. 450 days) but might be due to interactions between spices and proteins/enzymes which may lead to less protein leaking out into the TSp brine. SC contained 23.22 ± 0.59 wt% and 14.58 ± 0.56 wt% of DM and ash, respectively, and thus the DM content in this brine was similar to that of TSp, even though the curing time was shorter for SC; approximately 200 days vs. 450 days for the TSp. These results are in agreement with previous reports showing that both DM and protein content in brines from marinated herring changes significantly within the first 200 days of the ripening period and thereafter these leveled off [16]. The generally high DM content found in the brines was expected as it has been shown that up to 30% of the raw herring weight is lost during marinating whole carcasses and fillets [15], and thus biomolecules leak into the brine during marination. The volume and concentration of biomolecules in the brines depend mainly on the raw fish composition, additives used, processing water quality and process operations. In contrast to the three brines not containing acetic acid (TSa, TSp and SC), VC had a significant ($p < 0.05$) lower DM and ash content; 13.16 ± 1.90 and 5.80 ± 0.46 , respectively. When acid is present and the pH is lower than the isoelectric point of muscle proteins, electrostatic repulsion results in solubilization of the proteins. But when salt is added, this prevents protein solubilization (salting-out effect) and the structure "tightens up", which results in an increased firmness of the herring muscle and dehydration [29]. Rodger et al. [30] have shown that acetic acid penetrates the herring muscle quicker than salt and together they cause an initial hardening of the tissue, while simultaneously the drop in pH will activate proteolysis which will

result in subsequent tissue softening. These processes are likely taking place in VC, resulting in less pronounced leakage of proteins into the brine, observed when compared to other brines.

The brines were analyzed for their total fatty acid content, and except for VC the total fatty acids ranged from 4.01 ± 0.28 to 8.92 ± 0.38 mg/mL (Table 2). The fatty acid analysis showed that VC contained a significantly ($p < 0.05$) higher amount of fatty acids (20.1 ± 2.5 mg/mL) compared to any of the other brines. This was expected as fatty acids are better solubilized under acidic conditions compared to pure water. Also, it seems that the two desalting brines contained the same level of fatty acids as the corresponding blood brines (TSa and TSp). The reason for this might be that a noteworthy amount of the fatty acids are transferred via the fish to the fresh water during the desalting step. It should be noted that the comparison between the four types of brines is not straightforward, as the fat content in herring can vary from 1.3% to 25.7% [31] and the fat content of the initial herring raw material used was not measured. Hence, even though herring producer prefers high-fat herring, variations occur among the supplied raw material, and between these four products which were produced from different herring batches.

The iron content was determined in the brines, and it was expected that TSp and TSa would contain the highest levels due to the presence of blood (hemoglobin, Hb) in the brine. Indeed, the level of iron was highest in these brines with 5.68 ± 1.14 mg/kg and 4.16 ± 0.20 mg/kg for TSp and TSa, respectively, and thus can be attributed to the Hb content. However, processing salt is also known to contain trace elements and can influence product quality and some of the iron might originate from the salt. The corresponding desalting brines contained only 0.75 mg/kg of iron each, which presumably are blood residues from the herring fillets together with contribution from the salt. The iron content found in SC and VC were 2.08 ± 0.46 mg/kg and 1.61 ± 0.12 mg/kg, respectively. These products are prepared directly from fillets compared to TSa and TSp and consequently were expected to contain ferly less blood and thus less iron.

Table 3 shows the protein content, nitrogen content and density of the six brines. High protein content was present in TSp, TSa and SC, with values of 56.74 ± 0.02 , 48.37 ± 0.02 and 41.66 ± 0.01 mg/

Brine	pH	Dry matter content, wt%	Ash content wt%	Salt content wt%	Iron content (mg/kg)	Total fatty acids (mg/mL)
VC	4.03 ± 0.00^e	13.16 ± 1.90^d	5.80 ± 0.46^c	5.55 ± 0.09^c	1.61 ± 0.12^b	20.1 ± 2.5^a
SC	5.82 ± 0.03^c	23.22 ± 0.59^c	14.58 ± 0.56^b	14.21 ± 0.16^b	2.08 ± 0.46^b	4.01 ± 0.28^c
TSa	5.77 ± 0.02^{cd}	37.88 ± 0.34^a	16.12 ± 0.23^a	15.71 ± 0.08^a	4.16 ± 0.20^{ab}	8.92 ± 0.38^b
TSp	5.72 ± 0.01^d	26.62 ± 0.09^b	16.12 ± 0.06^a	15.53 ± 0.11^a	5.68 ± 1.14^a	5.36 ± 0.28^{bc}
D-TSa	6.32 ± 0.01^b	8.27 ± 0.07^e	5.14 ± 0.04^c	4.94 ± 0.03^d	0.75 ± 0.39^b	8.13 ± 0.54^{bc}
D-TSp	6.96 ± 0.05^a	1.91 ± 0.04^f	1.02 ± 0.01^d	0.99 ± 0.01^e	0.75 ± 1.76^b	4.45 ± 0.13^c

Values are given as means ($n = 3$) \pm standard deviations (absolute values). By columns, letters indicate homogeneous values ($p < 0.05$).

Table 2: pH, dry matter, ash, salt, iron and fatty acid content of the different brines.

Brine	Soluble protein		Nitrogen wt%	Density mg/mL
	mg/mL	wt% [†]		
VC	9.34 ± 0.01^d	0.90	0.34 ± 0.04^c	1042 ± 11^c
SC	41.66 ± 0.01^c	3.64	0.66 ± 0.00^b	1145 ± 2.7^b
TSa	48.37 ± 0.02^b	4.21	1.09 ± 0.03^a	1149 ± 9.6^b
TSp	56.74 ± 0.02^a	4.80	1.04 ± 0.01^a	1182 ± 14^a
D-TSa	13.16 ± 0.01^d	1.26	0.33 ± 0.01^d	1048 ± 0.5^c
D-TSp	4.39 ± 0.01^e	0.43	0.07 ± 0.00^c	1026 ± 6.7^c

Values are given as means ($n = 3$) \pm standard deviations (absolute values). By columns, letters indicate homogeneous values ($p < 0.05$)

[†] The protein content in wt% is calculated from the density, and all the standard deviation is below three significant figures

Table 3: Total protein content, total nitrogen and density of the six brines.

mL, respectively. The desalting brines, D-TSp and D-TSa, had lower protein content with 4.39 ± 0.01 mg/mL and 13.16 ± 0.01 mg/mL, respectively, and VC had a protein content of 9.34 ± 0.01 mg/mL. The high content of proteins in TSa and TSp is in accordance with a former study from our group [32], in which a protein content of 49 ± 2.2 mg/mL was reported from traditional barrel-salted herring brine. Further, Svensson et al. [8] and Andersen et al. [16] have both reported a protein concentrations of 50-60 mg/mL in brines from traditional barrel-salted herring ripened for 180 and 400 days, respectively. According to our knowledge, protein content for SC has not previously been reported. The protein content in VC was the lowest and this was probably due to the fact that acid denatures the proteins and thus reduces the protein solubility [29,33,34].

In accordance with the protein content, the highest nitrogen content was found in TSa and TSp, while the lowest content was found in D-TSa and D-TSp (Table 3). Szymczak and Kolakowski [9] showed that the amount of total nitrogen diffusing from herring meat into the brine is large and significantly increases with ripening time. The same authors also found that the loss of total nitrogen from fillets was higher than that from whole herring, most likely due to the larger exposed tissue surface. Total nitrogen and protein content were used to deduce and estimate the amount of non-protein nitrogen in our samples (values from Table 3 and a Kjeldahl factor of 6.25). The values found by Szymczak [15] were higher than the levels found in this study for TSa and SC, which are reported here to be 4.16 g/kg and 0.78 g/kg for whole herring and fillets, respectively. As stated in the materials and methods, TSa and TSp are placed in the brine first as whole fish and subsequently as fillets, whereas SC and VC are filleted prior to brining, and for this reason it was expected that the level of biomolecules leaking from the herrings into the brine would be higher in TSa and TSp, with leaking of blood protein first and muscle protein in the later stage. Additionally, the longer ripening period for TSa and TSp may also explain the higher level of non-protein nitrogen in these samples [16].

The proteinaceous fractions that are extracted into the brine can be divided into free amino acids (AA), peptides and muscle proteins including sarcoplasmic and myofibrillar proteins [10]. The protein profile of the six brines is shown in Figure 1. All brines, but VC, showed protein bands between 200 and 14 kDa and heavy bands in the 50 to 35 kDa region. TSp and TSa showed similar profiles, which also matched the respective desalting brines, D-TSp and D-TSa. SC showed a different protein profile when compared to the other brines, with an intense fragment at approximately 45 kDa. VC showed no bands on the gel which might be due to a combination of small peptides in this brine (smaller than 14 kDa) and that the acid have precipitated the proteins in the fish, thus less proteins leak into the brine. The protein pattern found in this study for TSa is in agreement with previous published results of brines from traditional barrel-salted herring [16,17,30] and similar with that of wastewater from salted codfish [35]. Christensen et al. [17] analyzed the brine from traditional barrel-salted herring after two days ripening and found several bands between 50 and 30 kDa. Andersen et al. [16] showed that the actin band (42 kDa) was fading in the herring muscle as a consequence of protein degradation and solubilization, and simultaneously became more pronounced in the brine during ripening. A rather heavy actin band was found in all brines, except in VC, as seen in Figure 1. Andersen et al. [16] also showed that herring myosin (200 kDa) was degraded during ripening and that several protein bands, reported to be myosin fragments, simultaneously developed in the region 200 to 40 kDa. Others have reported degradation products of herring myosin at 155, 146, 138, 123, 105, 65 and 56 kDa fragments

[30], some of which seem to be present in the gel presented in Figure 1, even if further confirmation is needed.

Total amino acid composition

Besides the chemical characterization given above, it is also of interest to investigate the amino acids present in the brines, due to the fact that some amino acids have biological activities such as antioxidant activity. Indeed, Pampanin et al. [36] showed that peptides from fish origin have interesting antimicrobial, antioxidant and immunomodulatory properties. Table 4 shows the total amino acids in the six brines divided into essential and nonessential amino acids. The amount of essential amino acids varied and was dependent on the type of brine. For example, all the brines were rich in lysine, but lysine was more abundant in D-TSp, D-TSa and VC compared to the other brines. The opposite was seen regarding the amount of valine, which was much higher in TSp, TSa and SC compared to the other three brines. Leucine was also present in high amount in all the brines although in a lower amount in the two desalting brines (D-TSp, D-TSa). Regarding the non-essential amino acids, alanine, glycine and proline were present in higher amounts in TSp, TSa and SC than in VC, D-TSa and D-TSp. Both aspartic acid (+ asparagine) and glutamic acid (+ glutamine) were present in high amounts in all the brines and were representing approximately 30% of the amino acids in the two desalting brines.

Table 5 presents the free amino acids in the brines as essential and nonessential amino acids and as observed in the total amino acid profile, some brines had similar free amino acid profile, with grouping of TSa, TSp and SC as well as D-TSa, D-TSp and VC. For example, for the essential amino acids TSa, TSp and SC were rich in lysine and threonine compared to D-TSa, D-TSp and VC which were rich in valine. All the brines, particularly VC, were rich in leucine. Regarding the non-essential amino acids, D-TSp, D-TSa and VC were rich in aspartic acid (+ asparagine), glutamic acid (+ glutamine) and serine. High amounts of these amino acids have previously been shown in the brines from barrel-salted herring [37]. Several amino acids are known to have antioxidant property, hereunder histidine, tryptophan, glycine, alanine, cysteine and lysine [38-40]. Pampanin et al. [36] analyzed small peptides (≤ 10 amino acids) from residual herring material (frame) and found that peptides showing an antioxidant activity included glycine, proline, lysine, alanine, histidine and glutamic acid. Another study performed on whole herring hydrolysates demonstrated

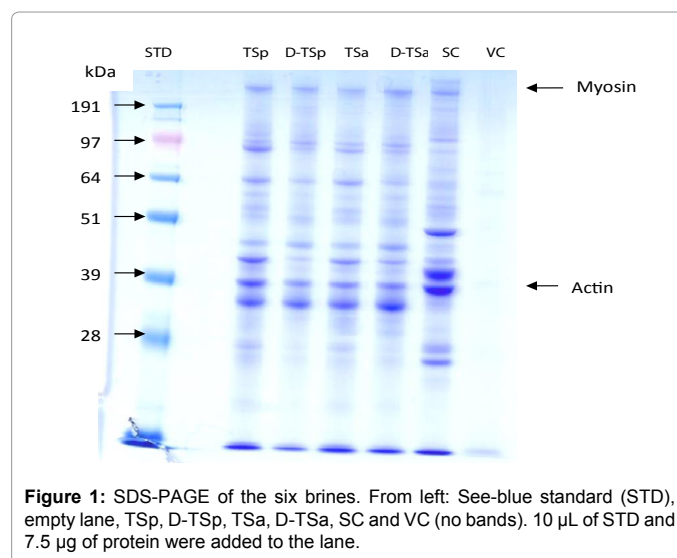


Figure 1: SDS-PAGE of the six brines. From left: See-blue standard (STD), empty lane, TSp, D-TSp, TSa, D-TSa, SC and VC (no bands). 10 μ L of STD and 7.5 μ g of protein were added to the lane.

Amino Acid	TSp	D-TSp	TSa	D-TSa	VC	SC
Essential						
Histidine	1.11 ± 0.04	1.73 ± 0.27	1.43 ± 0.00	1.49 ± 0.03	3.17 ± 0.55	2.53 ± 0.74
Isoleucine	2.76 ± 0.14	2.64 ± 0.89	3.90 ± 0.23	2.20 ± 0.21	2.47 ± 0.25	3.67 ± 0.16
Leucine	7.47 ± 0.56	5.57 ± 1.01	8.04 ± 0.22	6.22 ± 0.25	7.81 ± 0.72	8.61 ± 0.88
Lysine	9.68 ± 1.35	11.02 ± 0.90	9.85 ± 0.56	12.67 ± 0.64	14.95 ± 0.40	10.61 ± 0.90
Methionine	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Phenylalanine	2.76 ± 0.34	2.74 ± 0.12	2.87 ± 0.08	2.45 ± 0.04	3.69 ± 0.11	3.68 ± 0.51
Threonine	4.79 ± 0.21	4.06 ± 0.12	5.08 ± 0.06	3.88 ± 0.13	5.17 ± 0.01	4.87 ± 0.20
Tryptophan	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Valine	4.31 ± 0.08	1.69 ± 0.49	5.00 ± 0.16	2.68 ± 0.15	1.49 ± 0.44	4.26 ± 0.64
Total (a)	32.88	29.44	38.18	31.59	38.75	38.23
Nonessential						
Alanine	9.05 ± 0.74	n.d.	8.18 ± 0.83	4.52 ± 0.07	4.03 ± 0.10	7.65 ± 0.21
Arginine	9.04 ± 0.62	10.98 ± 3.47	5.74 ± 0.07	9.13 ± 1.08	5.52 ± 0.20	3.98 ± 0.79
Aspartic acid & Asparagine	11.33 ± 0.09	15.06 ± 1.01	11.84 ± 0.42	12.45 ± 0.35	13.42 ± 0.58	12.54 ± 0.12
Cysteine	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Glutamic acid & Glutamine	17.30 ± 1.97	30.72 ± 0.02	19.11 ± 2.13	27.94 ± 0.13	22.28 ± 0.16	19.67 ± 2.64
Glycine	8.62 ± 1.35	3.97 ± 0.63	7.55 ± 0.44	5.14 ± 0.30	6.28 ± 0.44	8.11 ± 0.79
Proline	3.33 ± 0.25	1.16 ± 0.31	4.03 ± 0.10	2.18 ± 0.11	1.92 ± 0.36	3.34 ± 0.32
Serine	7.16 ± 0.28	6.44 ± 0.31	6.06 ± 0.51	5.54 ± 0.09	5.71 ± 0.12	5.19 ± 1.00
Tyrosine	1.28 ± 0.15	1.71 ± 0.06	1.32 ± 0.05	1.51 ± 0.02	2.10 ± 0.10	1.29 ± 0.35
Total (b)	67.12	70.56	63.82	68.41	61.25	61.77
Grand total (a + b)	100	100	100	100	100	100
Grand total (g/L)	0.81 ± 0.09	0.12 ± 0.01	1.24 ± 0.06	0.61 ± 0.03	0.41 ± 0.12	0.66 ± 0.22

Values are in % of total amino acids (last row) in the brines and given as means (n = 3) ± standard deviations (absolute values). n.a. not analyzed; n.d. not detected.

Table 4: Total amino acid profile of the six different brines.

Free Amino Acid	TSp	D-TSp	TSa	D-TSa	VC	SC
Essential						
Histidine	1.32 ± 0.33	1.86 ± 0.55	1.47 ± 0.21	3.41 ± 1.19	3.78 ± 0.27	3.09 ± 1.01
Isoleucine	2.70 ± 0.08	2.78 ± 0.28	3.78 ± 0.40	3.68 ± 0.09	4.03 ± 0.28	3.37 ± 0.13
Leucine	7.05 ± 0.49	6.97 ± 0.77	8.28 ± 0.30	7.92 ± 0.19	11.65 ± 0.27	9.88 ± 0.31
Lysine	7.46 ± 0.24	10.99 ± 0.50	7.49 ± 0.46	10.49 ± 0.74	11.45 ± 0.58	9.69 ± 0.24
Methionine	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Phenylalanine	2.86 ± 0.01	3.89 ± 0.38	3.14 ± 0.03	4.34 ± 0.61	4.85 ± 0.39	4.77 ± 0.33
Threonine	3.78 ± 0.47	5.46 ± 0.40	4.59 ± 0.12	5.72 ± 0.53	6.02 ± 0.27	4.46 ± 0.22
Tryptophan	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Valine	4.29 ± 0.54	3.16 ± 0.10	6.32 ± 0.69	3.98 ± 0.59	2.94 ± 0.39	5.37 ± 0.08
Total (a)	29.54	35.19	35.05	39.54	44.72	40.63
Nonessential						
Alanine	7.92 ± 0.31	2.88 ± 1.38	10.61 ± 0.38	4.88 ± 0.88	7.43 ± 0.74	12.14 ± 0.44
Arginine	38.13 ± 1.98	29.45 ± 1.00	25.64 ± 2.00	20.63 ± 0.08	10.65 ± 0.82	18.47 ± 0.31
Aspartic acid & Asparagine	4.41 ± 0.24	8.80 ± 0.26	5.91 ± 0.07	8.23 ± 0.00	6.91 ± 0.40	5.91 ± 0.35
Cysteine	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Glutamic acid & Glutamine	5.72 ± 0.27	9.87 ± 0.87	8.25 ± 0.35	10.64 ± 0.15	12.70 ± 0.56	5.61 ± 0.20
Glycine	6.11 ± 0.92	2.88 ± 0.68	5.95 ± 0.46	4.52 ± 0.26	6.97 ± 0.06	10.28 ± 0.45
Proline	1.80 ± 0.01	1.94 ± 0.00	1.90 ± 0.10	1.50 ± 0.01	1.92 ± 0.19	1.35 ± 0.02
Serine	4.77 ± 0.88	6.59 ± 0.37	4.96 ± 0.19	7.44 ± 0.39	5.97 ± 0.10	3.58 ± 0.13
Tyrosine	1.59 ± 0.01	2.40 ± 0.31	1.73 ± 0.09	2.61 ± 0.27	2.72 ± 0.31	2.03 ± 0.08
Total (b)	70.46	64.81	64.95	60.46	55.28	59.37
Grand total (a + b)	100	100	100	100	100	100
Grand total (g/L)	0.40 ± 0.02	0.09 ± 0.02	0.48 ± 0.01	0.11 ± 0.00	0.02 ± 0.00	0.10 ± 0.01

Values are in % of total free amino acids (last row) in the brines and given as means (n = 3) ± standard deviations (absolute values). n.a. not analyzed.

Table 5: Free amino acid profile of the six different brines.

that antioxidant activities of the hydrolysate were associated with high amounts of glutamic acid, aspartic acid, glycine, alanine, leucine and arginine [12], which were also found in the brines of this study.

Antioxidant activity in the brines

In an attempt to further characterize the brines; three *in vitro* tests

were used to determine their antioxidant activity. As seen in Figure 2, all the brines exhibited good iron chelating activity. VC had the lowest activity (~55%), TSp reached ~70% activity, whereas the other four brines had activity between 80% and 90%. Interestingly, the desalting brines had very good iron chelating properties indicating that low concentration of low molecular weight compound present in the desalting brines might be acting as good chelators. Sannaveerappa et al. [41] reported that organic acids may play a major role in the antioxidant activity of herring press juice. Taheri et al. [32] previously fractionated the brine from traditional barrel-salted herring and analyzed the different fractions with the same assay, but found only negligible activity compared to EDTA. The higher iron chelating activity found in our study might be explained by the much higher protein and salt contents in our unfractionated brines.

The reducing power of the six brines is presented in Figure 3.

SC, TSa, TSp, VC and D-TSa showed similar or higher reducing power compared to the positive control, and D-TSp showed a poorer reducing power. The graphs in Figure 3 clearly show a concentration dependency of the reducing power, a dependency that has been showed before in herring salt brine [30]. Farvin et al. [23] studied the reducing power of yoghurt peptides and reported that it was the lower molecular weight fractions (3-10 kDa and <3 kDa) that contained the compounds with the highest reducing power. Fractionation was not done in our experiment so it is not possible to conclude on molecular weight of the antioxidant fraction and its exact nature. Sannaveerappa et al. [41] showed that press-juice from herring muscle efficiently prevented lipid oxidation in Hb-enriched washed cod muscle mince; with most of the antioxidant activity being located in the <1 kDa fraction.

Figure 4 illustrates that all the crude brines had the same, or higher, ability to scavenge ABTS-radicals as 2.5 mM Trolox, with TSa and

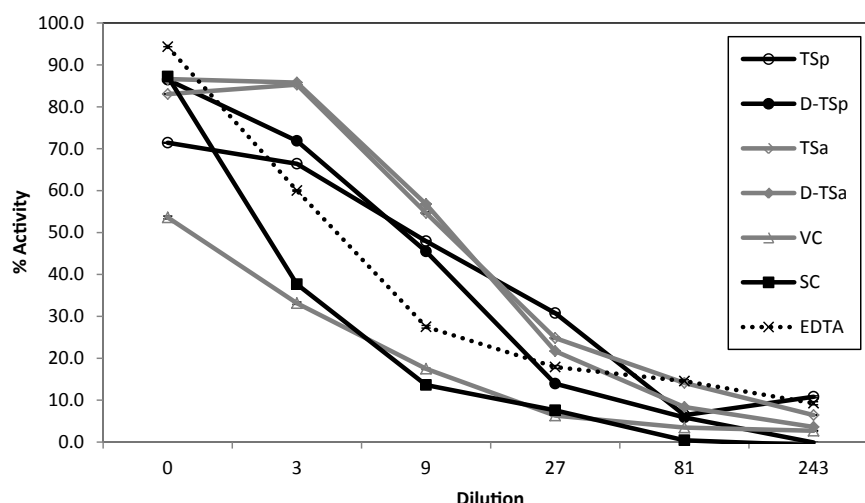


Figure 2: Iron chelating activity (%) in the six brines. The activity of the samples is plotted against the dilution factor (0-243). Control consisted of 200 μ M EDTA. Error bars indicates 95% confidence interval.

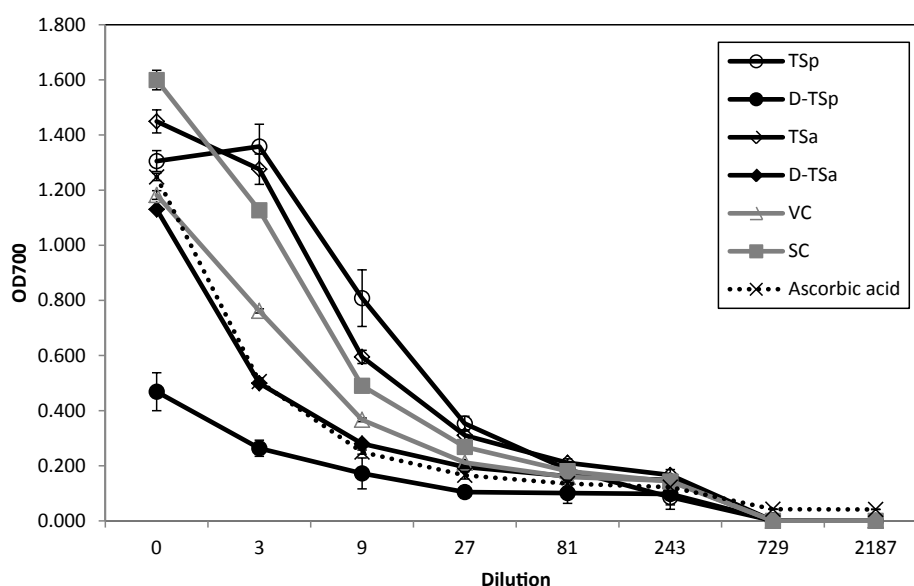


Figure 3: Reducing power (OD_{700}) in the brines. The activity of the samples is plotted against the dilution factor (0-2187). Control consisted of 500 μ M ascorbic acid. Error bars indicates 95% confidence interval.

TSp being the most potent samples. Dilution of the brines decreased the radical scavenging ability, thus showing a clear concentration dependency. The ability of protein-free fractions from herring salt brine to scavenge radicals in a concentration dependent manner has previously been shown by others [32], who showed no efficient DPPH-radical scavenging activity using this probe. This is in contrast with our results using ABTS as the probe showing that the results may be depending on the assays used.

The antioxidant effect of a hydrolysate of soybean protein was shown, by Chen et al. [42], to be mostly associated with short peptides (5-16 amino acids) that contain hydrophobic amino acids, valine and leucine, at the N-terminus, and proline, histidine or tyrosine in their amino acid sequence. In a previously published review by Freitas et al. [43] these same amino acids were, together with the sulfur containing amino acids, reported to provide antioxidant activity. All of the brines tested here contained these amino acids (Table 4). In some case the desalting brine also had very good antioxidant activity indicating that low concentration of low molecular weight compound, which are easily diffusing from the muscle to the brine, are potent antioxidants.

Antioxidants can be beneficial to human health as they may protect the body against Reactive Oxygen Species (ROS), which can modify membrane lipids, proteins and DNA and which are implicated in many diseases such as cardiovascular conditions, diabetes, cancer and Alzheimer's. Dietary intake of natural antioxidants could be an

important defense mechanism against ROS [44] and the present results show a potential of turning herring process water into value added products such as dietary supplements. The brines might also have good potential as food additives in order to prevent oxidation. The three tested antioxidant activities can, according to different mechanisms, prevent oxidation in food. Nevertheless, the classic *in vitro* tests for measuring antioxidant activity may not reveal the exact antioxidant potential of the brines in foods, and thus, tests in different food matrices should also be conducted [43]. Based on Figures 2-4, the most promising brines for further testing the antioxidant activity is the two blood-rich brines, TSa and TSp, together with SC and future studies will reveal whether the activity is solely in the protein-free fraction or if protein/peptides contribute to their antioxidant activity.

Enzymatic activity in the brines

The peroxidase activity in the brines is presented in Table 6. TSa and TSp showed a clear activity, with values of 2.20 ± 0.19 and 0.92 ± 0.04 Δ abs/min/ml, respectively. D-TSa, D-TSp, VC and SC did, on the other hand, not reveal any peroxidase activity. It was expected to find peroxidases in TSa and TSp as these two brines are rich in blood and consequently Hb, which is known to have pseudo-peroxidase activity [45]. However, the presence of other peroxidase enzymes is not ruled out, but requires further characterization.

The general protease activities measured as caseinolytic activity

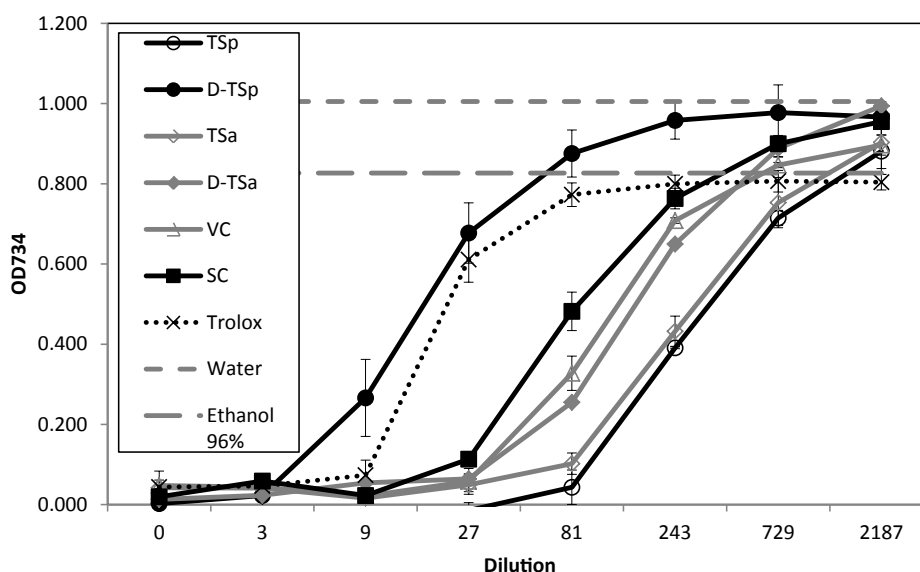


Figure 4: Radical scavenging activity (OD_{734}) in the brines. The activity of the samples is plotted against the dilution factor (0-2187). Control consisted of 2.5 mM Trolox. Error bars indicates 95% confidence interval.

Brine	Peroxidase activity Δ Abs/min/ml	Protease activity	
		Δ RFU/min/ml	Assay pH
VC	n.d.	6.81 ± 1.86^b	7.49
SC	n.d.	4.23 ± 0.67^b	7.25
TSa	2.20 ± 0.19^a	11.51 ± 2.04^a	7.25
TSp	0.92 ± 0.04^b	3.75 ± 1.55^b	7.15
D-TSa	n.d.	11.24 ± 1.22^a	7.61
D-TSp	n.d.	4.40 ± 1.55^b	7.71

Values are given as means ($n = 3$) \pm standard deviations (absolute values)
n.d., not detected.

Table 6: Peroxidase and protease activity in the six brines.

in the six brines are shown in Table 6 and show marked differences between brines. Some of the differences may be explained by differences in final pH during assaying which ranged from 7.15 – 7.71. As the brines will contain different types of exo- and endopeptidases with different pH optima [10], the final assaying pH can influence the total activity in the brine. Protease activity was highest in TSa and D-TSa. The fact that D-TSa contained the same level of protease activity as TSa was very surprising even though the final assay pH was slightly higher for D-TSa (7.61) compared to TSa (7.25). It is noteworthy that desalting for one day in fresh water will provide the same level of protease activity as nearly two years in the blood brine. This indicate that only a part of the proteases has been extracted during ripening and that considerably amount of enzymes is still present in the tissues and can be extracted during desalting. The higher activity in TSa compared to SC and VC was expected due to presence of viscera in the herring during ripening and thereby the contribution from intestinal proteases. It was therefore also noteworthy that TSp contained only approximately 1/3 of the activity of TSa. Since TSa and TSp were both prepared from whole herring, enzymes from the intestinal and digestive track are expected to leak out into the brine, and we would have expected similar enzyme activity in these two brines. The main difference between TSa and TSp are the spices and it cannot be excluded that spices contain compounds that can have protease inhibiting properties, and that some of the added spices are influencing the activity of the proteases in the brine. However, it is also possible that the difference is mainly caused by batch variation. The analyzed brines are from a conventional industry, thus the herrings that resulted in TSa and TSp are not caught at the same place or processed at the same time, thereby not from the same raw material, and thus might represent different digestive enzyme activity. We have analyzed samples representing a snapshot of the actual industry and not from controlled ripening experiments. This is also supported by protease activity measured in D-TSp which was at the same level as TSp and thereby exhibited the same pattern as for TSa and D-TSa. This also support that proteases can be extracted during the desalting process. The protease activity in VC was $6.81 \pm 1.86 \Delta\text{RFU}/\text{min}/\text{ml}$, and thus, the second highest. This moderately high activity may be explained by the low pH in the VC brine (4.03) which might activate some muscle proteases to a greater extent than the higher pH in the other brines (5.72 – 6.96), especially SC where the activity also originates from muscle proteases. However, to elaborate more specifically on the exact nature of the exo- and endopeptidases responsible for the protease activity in the different brines, more specific investigations are needed [10,46].

This study has shed light on the production of four different marinated herring products and the enormous amounts of process water generated during this production. These brines are currently discarded as waste prior to packaging of the end-product. The chemical characterization of the six brines revealed that they are very rich in dry matter, salt, protein, non-protein nitrogen, iron and fatty acids. In fact, with more than 200 L brine per 100 kg herring produced, this could sum up to 4 kg of lipid and more than 11 kg of protein. In addition, our investigation has demonstrated that there is a huge potential for extracting compounds of high marked value from these herring by-products, due to the antioxidant and enzymatic activity reported here. The desalting brines showed to be promising sources of antioxidants and enzymes. The use of natural antioxidant extracts to replace synthetic antioxidants in foods is currently in focus, and as such herring brine can become an interesting novel food ingredient. However, more studies are needed to further characterize the precise nature of the antioxidants and enzymes and to investigate their potential application in foods or their conversion into compounds with good commercial value.

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PAPER II

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Recovery of biomolecules from marinated herring (*Clupea harengus*) brine using ultrafiltration through ceramic membranes



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Clupea harengus

ABSTRACT

Marinated herring processing brines, which are usually discarded, are rich in salt, protein, non-protein nitrogen, iron, fatty acids, antioxidant and even possess enzymatic activity. This study investigated the performance of ceramic ultrafiltration of two herring spice brines with a major focus on recovery of high value biomolecules such as proteins, fatty acids, minerals, and phenolic compounds. Chemical and biological oxygen demand (COD, BOD₅) as well as total suspended solids (TSS) were also measured to follow the performance of the ultrafiltration. The retentates contained 75–82% (<62.7 mg/mL) of the protein and 75–100% of the fatty acids compared to the level in the initial brines. The nitrogen concentration was approximately halved in the permeate, whereas the phosphorous content was significantly increased in the permeate compared to the initial brines. Moreover, a retention of up to 42% COD, >95% TSS and >85% iron was obtained using the ceramic membranes. The two permeates generated were both fat-free and contained approx. 2% of the proteins compared to the unfiltered brines, and the retention of the phenolic compounds were ranged from 0 to 39%. The results presented in this work demonstrate that ceramic ultrafiltration can recover biomolecules from marinated herring brines although pre-filtration optimization is still needed.

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1. Introduction

In the production of marinated herring, a traditional Scandinavian product, the volumes of wastewater generated can reach more than 700 L per 100 kg herring produced; of which up to 100 L is generated as brine during the final maturation step. This wastewater is usually pooled and treated at the “end of the pipe” without any attempt to collect the organic matter or recycle the water. The costs for disposal of waste and wastewater and the environmental regulations are two major driving forces for introducing separation and treatment technologies. However, the recovery of (high) valuable biomolecules for human, animal or industrial uses is, in many cases, only the secondary purpose (Waldron, 2007), but have recently received increasing focus. In a review by Olsen, Toppe, and

Karunasagar (2014) the use of by-products as new resources is discussed and it is stated that today only a few high-value products are on the market. They point at an overestimation of the market, small volumes of high quality by-products, and high cost related to purifying biomolecules of interest. Nonetheless, before they even reach the industrial waste streams, marinated herring brines are food grade and follow hygienic standards. Therefore, it might be realistic to extract valuable compounds from these liquid effluents.

In the treatment of wastewater, a promising technique is membrane filtration; either ultrafiltration (UF) alone or in combination with e.g. microfiltration (MF) (Afonso, Ferrer, & Bórquez, 2004). Using UF, one can separate high molecular weight (HMW) components, like proteins and suspended solids, from low molecular weight (LMW) components, like mono- and disaccharides, amino acids, inorganic acids, or sugars and salts (Wagner, 2001). Afonso et al. (2004) studied an integrated process of MF and UF for the concentration of proteins from fish meal effluents, which resulted in a recovery of 69% of the proteins. Such recoveries may

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allow for additional revenue and a significant reduction of environmental pollution. Cassini, Tessaro, Marczak, and Pertile (2010) demonstrated that the use of ceramic UF in the pre-treatment of isolated soy protein wastewater has great potential, as they managed to retain 34% chemical oxygen demand (COD), 52% protein, and 86% total suspended solids (TSS).

Ceramic membranes made from silicon carbide (SiC) have recently been certified for use in the food industry, and according to the manufacturer these membranes are extremely hydrophilic, thus very efficient in oil/water separation. Besides this, they are chemically inert and tolerate high salt content and temperature. With these properties, ceramic membranes might offer a better separation of the herring brines, compared to traditional synthetic polymer membranes. One serious problem in UF is membrane clogging and pre-treatment is therefore usually required. Electroflocculation (EF) is an electrochemical pre-treatment method, which has been shown to be a low-cost technique in e.g. harvesting marine microalgae (Lee, Lewis, & Ashman, 2013) and in separating solid and dissolved pollutants from wastewater (Mollah, Schennach, Parga, & Cocke, 2001). EF utilizes electric current through the use of a 'sacrificial' anode that releases iron or aluminium ions through electrolytic oxidation. These coagulant ions lead to aggregation of suspended particulate matter, and as the process also produces hydrogen bubbles the result is flotation of flocs (Ben-Sasson & Adin, 2010). Some of the reported advantages of EF are clear, colourless and odourless water, easy separation of flocs, and no need for chemicals (Mollah et al., 2001).

In an attempt to separate and collect high valuable biomolecules from marinated herring brines into HMW and LMW fractions, we have here combined EF with UF to evaluate if this could provide a feasible and unique separation setup that has not been previously tested within seafood processing. In a recent study we analysed brines from four different marinated herring products, and showed that they are rich in dry matter, salt, protein, non-protein nitrogen, iron, fatty acids, antioxidant activity as well as peroxidase and protease activity (Gringer, Osman, Nielsen, Undeland, & Baron, 2014). Two brines contained spices, and although the composition of the spice mixture is confidential, it is likely that the documented antioxidant activity was a result of spice-derived polyphenols (Shahidi & Zhong, 2010). For this reason, the brines tested in this work were from spice-cured (SC) and traditional barrel-salted spice-cured (TSp) herring products.

The objective of the present work was thus to evaluate the performance of either EF or a synthetic polymer membrane i.e. a polypropylene filter (PF) pre-treatment in combination with ceramic UF for the fractionation of TSp and SC into HMW retentate and LMW permeate and to investigate the partitioning of the high value biomolecules into these fractions; e.g. proteins, fatty acids, and phenolic compounds whilst water quality indicators were also monitored (COD, BOD₅ (biological oxygen demand in 5 days at 20 °C) and TSS).

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade, purchased from Merck (Darmstadt, Germany) or Sigma–Aldrich (Steinheim, Germany). All water used was double deionized water.

2.2. Treatment of samples

2.2.1. Herring brine

Brines from the last step in the production of marinated herring (*Clupea harengus*) were supplied from Lykkeberg A/S (Hørve,

Denmark), and prepared according to Lykkeberg A/S production protocols. Two brines were obtained; from spice-cured fillets (SC), and from traditional barrel-salted spice-cured herrings (TSp). For SC, fillets were ripened for ~190 days. TSp results from salting whole herring for 200 days whereafter filleting takes place and the fillets are placed back in the brine for additional ripening (~1 year). The brines have been previously characterised (Gringer et al., 2014) and contain 14–16% salt, 41–57 mg/mL protein and 4–9 mg/mL lipids. Furthermore, they also contain a considerable amount of sugar and spices, however the levels are confidential. Brines were treated on-site using the tested technologies and generated samples were stored at –80 °C until further analysis.

2.2.2. Electroflocculation (EF) and polypropylene filter (PF)

For EF, a pilot scale unit (Fig. 1, point (3)) was provided by Application Factory A/S (Hørsholm, Denmark), consisting of a polypropylene housing with inlet at one bottom corner and outlet at the opposite top. Inside, a number of aluminium plates were placed, through which the process water was flowing. Flow rate was 1000 mL/min and 180A was applied. Foam/floc were produced which was manually collected. The outlet of EF (EF-OUTLET) was drained 5 cm from the bottom (to avoid the sediment particles) into the collecting tank (4), and used as inlet for the UF unit.

For membrane filtration, a dead-end 50 µm polypropylene filter (PF) (6) was used (Heco Filtration A/S, Hedensted, Denmark). Filtration was carried on until complete clogging of the filter, ~2 h. Outlet of PF unit was retentate (PF-RET) and permeate (PF-PER), of which the latter was inlet to UF.

2.2.3. Ultrafiltration (UF)

UF tubular ceramic membranes made of silicon carbide (SiC) were supplied by LiqTech International A/S (Ballerup, Denmark). The membrane was Ø 25 mm × 305 mm with 31 × 3 mm round channels. Filtration area was 0.09 m², and nominal pore size 0.040 µm. This system, shown in Fig. 1, consisted of a collecting tank (100 L) (4), a recirculating pump (7), and the UF membrane inside a stainless steel housing (9). The feed solution (EF-OUTLET or PF-PER) flowed along the length of the membrane and was divided into permeate (13), or retentate which returned to the collecting tank (4). The system operated in cross flow mode (2 m/s) with three pressure transmitters (0–6 bar), placed at the module entrance (8), at the concentrate outlet (10) and at the permeate outlet (12). The transmembrane pressure was controlled by the retentate valve (11) and the recirculating pump (7), and was kept at 2–2.6 bar. Process time was kept at exactly 1 h, after which both permeate (UF-PER) and retentate (UF-RET) was collected. During UF the temperature increased from 5/7 °C to 24/26 °C for the different brines. Efficiency of the membrane is described by the retention (%), *R*, given by the difference in the concentration (*C*) of the compound of interest in permeate and in inlet water:

$$R = \left(1 - \frac{C_{\text{permeate}}}{C_{\text{inlet}}} \right) \cdot 100\%$$

2.3. Methods

2.3.1. Proximate composition

pH was measured using a Metrohm 827 pH-meter (Herisau, Switzerland). Salt content (wt%) was measured according to AOAC standard method (2005). Dry matter (DM) content was measured by a two-step evaporation of water (60 °C for 24 h plus 105 °C for 24 h), and calculated as (mass of dry sample*100)/(mass of wet sample). Ash content (%) was measured by burning at 600 °C for

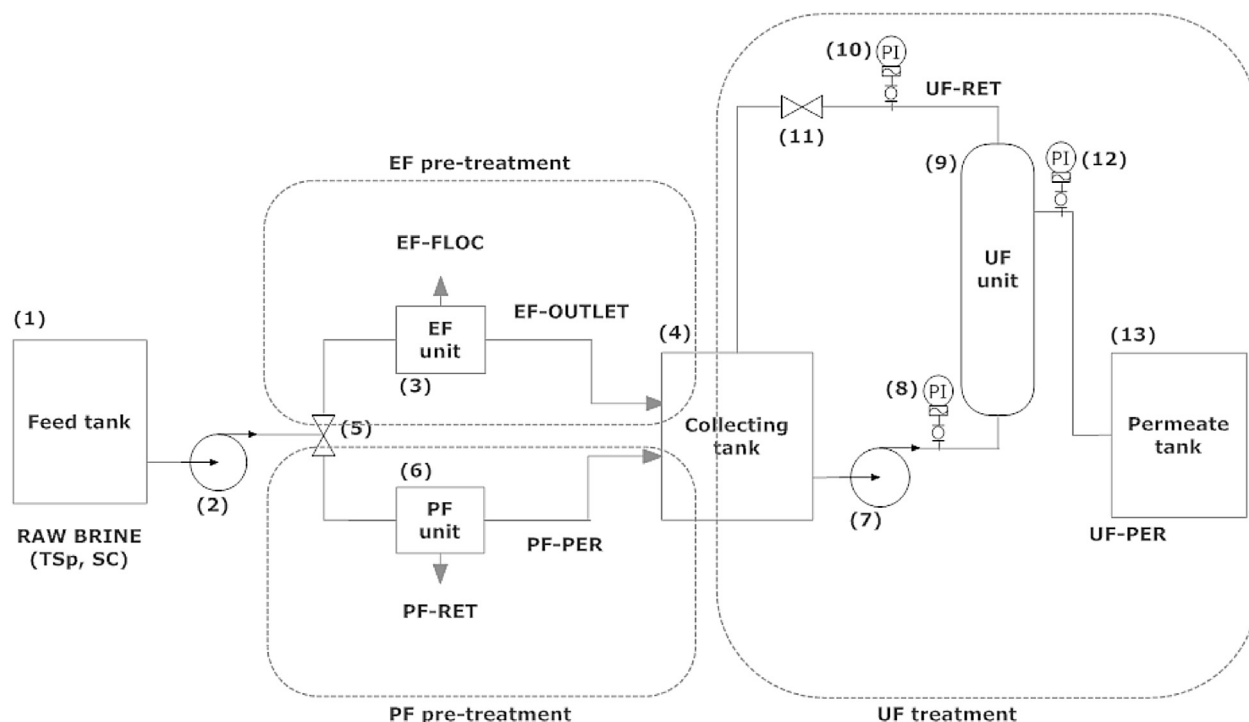


Fig. 1. Flow chart of the pilot scale units. EF = electroflocculation, PF = polypropylene filter, UF = ultrafiltration. (1) Feed tank containing either TSp or SC, (2) feed pump, (3) EF unit, (4) collecting tank for either EF-OUTLET, PF-PER or UF-RET, (5) valve, (6) PF unit, (7) recirculation pump, (8) pressure transmitter, (9) UF unit, (10) pressure transmitter, (11) valve, (12) pressure transmitter, (13) permeate tank for UF-PER.

24 h, and reported as (mass of ash*100)/(mass of wet sample). Protein content (mg/mL) was obtained using the BCA kit (Thermo Scientific, Pierce®, Rockford, USA), with bovine serum albumin (BSA) as standard. Fatty acid analysis was completed by extraction with chloroform and methanol (Lee, Trevino, & Chaiyawat, 1996) followed by methylation and detection by gas chromatography mass spectrometry, GC-MS (Cavonius, Carlsson, & Undeland, 2014). Chromatographic parameters were previously described by Gringer et al. (2014). Total fatty acid (mg/mL) was calculated as the sum of all detected fatty acids, using C:17 as standard.

2.3.2. Trace elements

In the analysis of iron, zinc, calcium and magnesium, 0.75 mL of concentrated nitric acid, 0.15 mL of concentrated hydrochloric acid and 5 mL sample were mixed in Teflon vials. Samples were digested (Ethos plus milestone microwave, Sorisole, Italy) with a temperature increase from 20 to 180 °C in 15 min and subsequently kept at 180 °C for 20 min. Samples were cooled to room temperature, decanted into test tubes and diluted to 10 mL using water. Analysis of iron and zinc was conducted by ion chromatography according to Fredrikson, Carlsson, Almgren, and Sandberg (2002). Analysis of calcium and magnesium was performed using Atomic Absorption Spectroscopy (Agilent Technologies, Kista, Sweden). Quantification was conducted using iron and zinc standards (Fluka, Buchs, Switzerland) and calcium and magnesium standards (Ultra Scientific, Rhode Island, USA), and reported in µg/g. Nitrogen content (mg/g) was determined by Kjeldahl method, according to AOAC standard method (1995). Total phosphorous content (mg/L) was measured by photometric cuvette tests from Hach Lange (LCK349, Brønshøj, Denmark) and measured on a high-performance VIS spectrophotometer at 800 nm (DR3900, Hach Lange, Denmark). Aluminium content was measured by complete digestion and subsequent analysis by inductively coupled plasma mass spectrometry, ICP-MS (Perkin–Elmer SCIEX, ELAN 6000). For sample

digestion, 2 g of brine was mixed with 5 mL HNO₃ (69%), 3 mL H₂O₂ (30%) and 0.5 mL HCl (37%), and digested (10 min) in a microwave (Multiwave 3000, Anton Paar, Austria) at 1400 W, according to Anton Paar Application Notes. Prior to ICP-MS analysis water was added to a final volume of 10 mL. Aluminium content (mg/L) was calculated from a standard curve with CentiPUR Aluminium standard solution (Merck, Darmstadt, Germany).

2.3.3. Water quality indicators

COD and BOD₅ were determined by photometric cuvette tests from Hach Lange (LCK014 and LCK555, respectively), measured on the DR3900 spectrophotometer mentioned above at 605 and 620 nm, respectively and reported in g/L. TSS were determined according to Danish Standard (1985), and reported in g/L.

2.3.4. Phenolic compounds and oxidation

Total phenolic compounds (TPC) were determined according to Farvin and Jacobsen (2013). In short, 100 µL brine was mixed with 0.75 mL 10% Folin-Ciocalteu reagents and incubated at room temperature for 10 min. 0.75 mL 6% sodium bicarbonate was added and incubated at room temperature for 90 min in the dark. Subsequently, the samples were vortexed and centrifuged (3 min at 13,684 g) and the absorbance read (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instruments, Inc., Vermont, USA) at 725 nm on 200 µL. A calibration curve was made with gallic acid (GA) and TPC is reported as mg gallic acid equivalents (GAE)/mL brine:

$$\frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) - A_{0\mu\text{g GA/mL}}}{\text{slope of calibration curve}} \cdot \text{dilution factor}$$

Oxidation was measured by determining the concentrations of thiobarbituric acid reactive substance (TBARS) according to Sørensen and Jørgensen (1996) and adjusted for sugar (Du & Bramlage, 1992). This method is preferred as it gives a good

general indication of the oxidative status and it is known to correlate well with sensory evaluation. In short, 5 mL of supernatant (3 min at 13,684 g) were homogenized with 30 mL of 7.5% trichloroacetic acid (TCA) containing 0.1% propylgallate (PG) and 0.1% ethylenediaminetetraacetic acid, disodium salt (EDTA) for 30 s in an Ultra Turrax blender (9500 rpm, IKA® T25, Staufen, Germany) and filtered through a Whatman filter no. 42. Five mL of the filtrate was mixed with 5 mL 0.02 M thiobarbituric acid (TBA) solution and incubated at 100 °C for 40 min, before reading the absorbance at; 440, 532 and 600 nm, using ϵ at 532 nm of 1.57×10^5 (Albro, Corbett, & Schroeder, 1986) for malondialdehyde (MDA), and the molar absorbance (MA) of sucrose at 440 and 532 nm of 147 and 8.4, respectively (Du & Bramlage, 1992). Nonspecific turbidity was extracted at 600 nm. TBARS values are given as MDA equivalents (MDAeq) in nmol MDAeq/mL brine:

$$\frac{(A_{532} - A_{600}) - (A_{440} - A_{600}) \cdot \left(\frac{MA_{532 \text{ of sucrose}}}{MA_{440 \text{ of sucrose}}} \right)}{157000} \cdot 10^6$$

2.4. Statistical analysis

Measurements were carried out in triplicates unless otherwise stated. Results are given as means \pm standard deviations. The GraphPad Prism® software Ver. 4.03 was used with $P < 0.05$ and results were compared using one-way ANOVA with Tukey's post-test.

3. Results and discussion

3.1. Pre-treatment

Electroflocculation (EF) with aluminium ions or membrane filtration with a dead-end 50 μ m polypropylene filter (PF), were evaluated as methods of pre-treatment of TSp and SC prior to ceramic UF in order to analyse the partitioning of the high value biomolecules in HMW retentate and LMW permeate.

For EF-tests with TSp, 73 kg TSp RAW BRINE was used of which 20.3 kg was dry matter, 10.4 kg was ash, 5.1 kg was protein and 0.7 kg was fatty acids. The retention of molecules provided by EF is given in Table 1 and show, that dry matter, ash and protein were retained to the same degree; 47, 46 and 49%, respectively. The fatty acids were, however, retained to 79%. Running SC through the same pre-treatment, the amount of SC RAW BRINE used was 119.4 kg, of which 28.8 kg was dry matter, 17.3 kg was ash, 6.3 kg was protein and 0.3 kg was fatty acids. In comparison with EF-treatment of TSp, subjecting SC to EF gave rise to slightly higher retentions regarding dry matter, ash and protein, 55–58%, whereas the fatty acid content was retained to the same extend. Testing with PF, the starting amounts were 37.6 kg and 37.0 kg RAW BRINES TSp and SC, respectively, and the compositions the same as in the EF-trials. In

Table 1

Retention (%) of dry matter, ash, protein and fatty acids content in EF-OUTLET and PF-PER after pre-treatment with either electroflocculation (EF) or a polypropylene filter (PF) in TSp and SC brine.

	TSp		SC	
	EF-OUTLET	PF-PER	EF-OUTLET	PF-PER
Dry matter	47%	36%	55%	37%
Ash	46%	35%	56%	34%
Protein	49%	34%	58%	35%
Fat	79%	51%	78%	79%

Values are calculated on mean ($n = 3$) values from inlet and permeate samples.

both cases, the retention of dry matter, ash and protein were between 34 and 37%. The retention of fatty acids was 51% and 79% for TSp and SC, respectively. The amounts of EF-OUTLET and PF-PER, were approximately 49% and 67%, of starting RAW BRINES, respectively, for both TSp and SC, and thus EF resulted in less inlet for the subsequent UF treatment compared to PF. On the other hand, it should be noted that EF was more time efficient than PF. Others have shown that EF is a time efficient and low cost technique, for example Ben-Sasson and Adin (2010), who reported a significantly decrease (up to 90%) in filtration-energy consumption in dead-end microfiltration using aluminium-based EF as pre-treatment. Xu, Sheldon, Larick, and Carawan (2002) tested aluminium-based EF on the wastewater from egg processing for recovery and utilization of this by-product. They recovered high concentrations of protein (36–50%) and fat (32–42%) in the generated sludge/floc, which is comparable with the values found in our work, although the fatty acid retention was even higher for our brines (78–79%). Afonso et al. (2004) studied the protein recovery from fish meal effluents by microfiltration followed by ultrafiltration. During the microfiltration step (decreasing pore size: 80, 20 and 5 μ m) they obtained a retention of 19% protein and 94% fat. These results were thus 1.8 times higher (34–35%) in terms of protein retention and 1.1–1.8 times lower (51–79%) in terms of fat retention compared to our study, which may be due to different composition of effluents used in their study.

As described in Section 2.2.2, EF makes use of a 'sacrificial' anode that releases aluminium ions of which some end up as part of the flocs, whilst others will sediment at the bottom of the equipment or even solubilize in the EF-OUTLET. Exposure of aluminium has been described in connection with development of e.g. Alzheimer's disease (Bondy, 2014) hence is considered a health hazard. Additionally, in 2007 the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2007) developed a provisional tolerable weekly intake for aluminium of 1 mg/kg body weight. Thus, as the main purpose of our work was to evaluate the recovery of high value biomolecules from TSp and SC after pre-treatment with e.g. EF, it was necessary to investigate the level of aluminium ions in the EF-OUTLET. The aluminium content reported in Table 2 clearly shows that not all aluminium ended up in the floc or sediment during EF, but was solubilized in the EF-OUTLET and was present in both UF-PER and UF-RET in significantly ($P < 0.05$) higher concentrations than after PF. The highest concentrations were detected in UF-RETs after EF treatment of TSp and SC, with values of 1371 and 1688 mg/L, respectively. The amount of aluminium present in the permeates represent approximately 12–14% of the amount present in the retentates and was 5 and 25 times higher when compared to the PF-PERs for TSp and SC. On the basis of these results, EF using an aluminium electrode is not appropriate as a pre-treatment for recovery of high value biomolecules from marinated herring spice brines, thus only PF was further considered.

3.2. Ceramic ultrafiltration

As a second step after PF, UF was conducted with ceramic membranes. A mass balance was carried out to evaluate the

Table 2

Aluminium content (mg/L) in UF-PER and UF-RET after EF and PF pre-treatments.

	Pre-treatment	TSp		SC	
		UF-PER	UF-RET	UF-PER	UF-RET
Al (mg/L)	EF	175 \pm 18 ^a	1371 \pm 18 ^a	243 \pm 40 ^a	1688 \pm 15 ^a
	PF	38 \pm 10 ^b	93 \pm 5 ^b	10 \pm 7 ^b	33 \pm 10 ^b

Values are given as mean ($n = 3$) \pm standard deviation (absolute value). By columns, same letter indicate no statistically difference ($P < 0.05$).

consecutive treatment of PF and UF (Table 3). It shows that the UF-PERs (generated from 25.2 and 24.9 kg TSp and SC PF-PERs, respectively) only amounted to 1.2 and 1.6 kg, respectively, i.e. 4.8% and 6.4%, which is probably due to membrane clogging which also impacted the flux (~ 20 L/m²/h). Still the fatty acids were retained by 100% by the ceramic membrane, which was due to the hydrophilicity of the membrane. The retention of dry matter and ash was 95.5% and 94.3% for TSp and 94.7% and 94.3% for SC, respectively. Proteins were retained by 98.8% and 98.2% for TSp and SC, respectively. The reported dead volume in the pilot plant was approximately 5 L, but with a continuous process under industrial conditions no dead end volume would be expected.

Afonso et al. (2004) studied the protein recovery from fish meal effluents by consecutive microfiltration and ultrafiltration. The UF alone resulted in 62% retention of the proteins (15 kDa molecular weight cut-off (MWCO)), and the integrated process of microfiltration and UF enabled 69% retention of the proteins. Dumay, Radier, Barnathan, Berge, and Jaquen (2008) used a 10 kDa MWCO cellulose membrane for the recovery of proteins and lipids from sardine surimi wastewater and they obtained a retention of >70% protein and >90% lipids. In our study a much higher protein retention was obtained (>98%) even though the MWCO in the present work is expected to be > 200 kDa.

Investigation of the organic load in the fractions before and after PF and UF, using the water quality indicators COD, BOD₅, TSS and salt (Table 4), shows that the retention of COD was about 12% and 42% for TSp and SC, respectively. In respect to TSp, the COD level was almost unaltered after PF, whereas the PF retained almost 28% of the COD in the case of SC. The TSS level was reduced with more than 95% in both of the UF-PERs. A large part of the TSS was already retained by PF (approx. 51% and 22% for TSp and SC, respectively). There was no significant ($P < 0.05$) change in the BOD₅ level in any of the outlets for either TSp or SC, indicating that PF and UF are not affecting the biological matter in these brines. This might be due to the fact that smaller biomolecules, affecting BOD₅, are perfectly capable of permeating the 40 nm pore size of the ceramic membrane. Indeed, the BOD₅ is linked to the ability of biological organisms to break down organic material and since the brines contain compounds that are complex/difficult to break down this may explain the resilience of BOD₅. Cassini et al. (2010) studied ceramic UF of wastewater from isolated soy protein production. They reported retention of 26% and 85% of COD and TSS, respectively, which are data comparable to our results. In contrast to our results, a retention of about 80% BOD₅ was achieved by Mamari et al. (1996) who studied fishery washing water. Overall, the values found for COD, BOD₅ and TSS in TSp and SC were all higher than the general levels reported for all types of herring processing waters reviewed by Chowdhury, Viraraghavan, and Srinivasan (2010).

The content of trace elements, i.e. Zn, Fe, Mg, Ca, P and N is presented in Table 5, from which it is seen that there was no

significant ($P < 0.05$) difference in the concentration of zinc in the RAW BRINE, UF-PER and UF-RET for either of the two brines. Iron, on the other hand, was retained by 85% and 91% in SC and TSp, respectively. Magnesium was present at a significantly ($P < 0.05$) higher level in SC compared to TSp, with 533 $\mu\text{g/g}$ and 307 $\mu\text{g/g}$ in the RAW BRINES, respectively, and the concentrations were unchanged during PF and UF treatments. The concentration of calcium was remarkable, as it was slightly increased (yet not significantly) from 133 to 160 $\mu\text{g/g}$ in the UF-PER in the case of SC. However, it was significantly ($P < 0.05$) retained in the case of TSp (265–160 $\mu\text{g/g}$). To the best of our knowledge, the retention of Zn, Fe, Mg and Ca in UF treated herring brines has not been reported previously. The retention of some of the trace element may be due to complexation to protein.

The content of phosphorous in TSp and SC RAW BRINES was high; 2875 and 1880 mg/L, respectively (Table 5), and even higher in the corresponding UF-PERs; 3228 and 2188 mg/L, respectively. The concentration of phosphorous was higher in permeate compared to the inlet i.e. the retention was negative. Negative retention has previously been observed in UF studies i.e. when charged macromolecules are retained by the membrane, negative retention can be observed for the co-ions (Akred, Fane, & Friend, 1980; Noordman, Ketelaar, Donkers, & Wesselingh, 2002). Hence, the data in this study indicate that the retained macromolecules are negatively charged and the phosphorous retention is negative due to electrostatic repulsion by the negatively charged macromolecules.

PF and UF retained 59% and 44% nitrogen in SC and TSp, respectively, resulting in 0.35% and 0.81% nitrogen in the UF-PERs. Szymczak and Kolakowski (2012) studied the losses of nitrogen from herring to brine during marination. They found that herring meat contain 2.6–2.9% nitrogen of which 18–27% was lost during marination, thus values of 0.5–0.8% were expected to be found in the RAW BRINES. They observed that a higher nitrogen loss was obtained from fillets than from carcasses. In this study a significantly ($P < 0.05$) higher nitrogen content was found in TSp than in SC (1.44% vs. 0.86%) indicating that the carcasses might lose more nitrogen than the fillets and also that ripening time has a profound effect on nitrogen loss. This might be explained by the fact that even though TSp is a result of marination of carcasses, the herrings are subsequently placed back in the brine as fillets and thus not solely exposed in the brine as carcasses.

3.3. Recovery of high value biomolecules

The main goal of this work is to recover high value biomolecules from spice brines into HMW and LMW fractions. Due to the high market value of fish proteins and lipids and the increasing interest in natural antioxidants, the marinated herring brines can represent an untapped source of valuable biomolecules. Therefore, protein, fatty acids (FA), n-3-fatty acid (n-3-FA) and TPC were determined. The content of protein, FA, n-3-FA and TPC in TSp RAW BRINE were 65.1 mg/mL, 5.9 mg/mL, 1.1 mg/mL and 3.7 mg GAE/mL, respectively. In TSp UF-PER, these concentrations had decreased to 20.1 mg/mL, 0.05 mg/mL, 0.0 mg/mL and 3.8 mg GAE/mL, respectively. As seen in Fig. 2, the retention was close to 80% for protein, 100% for both FA and n-3-FA for TSp, whereas the TPC was not significantly ($P < 0.05$) retained by the membrane. SC RAW BRINE and UF-PER had protein contents of 45.3 mg/mL and 10.5 mg/mL, respectively. The FA and n-3-FA were reduced in the SC RAW BRINE and UF-PER from 5.0 mg/mL to 0.05 mg/mL and from 0.68 mg/mL to 0.0 mg/mL, respectively. The retention of protein, FA and n-3-FA was similar for SC and TSp. However, comparing the partitioning of phenolics in SC vs. TSp, there was a clear difference. In SC RAW BRINE there was 2.58 mg GAE/mL which decreased to 1.58 mg GAE/

Table 3

Mass balances based on consecutive PF and UF treatment of TSp and SC. Amount of brine, dry matter (DM), ash, protein and fatty acids (FA) is reported in kg.

	TSp				SC			
	Inlet		Outlet		Inlet		Outlet	
	PF-PER	UF-PER	UF-RET	Loss	PF-PER	UF-PER	UF-RET	Loss
Brine (kg)	25.2	1.2	19.3	4.7	24.9	1.6	18.2	5.1
DM (kg)	6.7	0.3	5.1	1.3	5.7	0.3	4.0	1.4
Ash (kg)	3.5	0.2	2.6	0.7	3.5	0.2	2.4	0.9
Protein (kg)	1.6	0.02	1.2	0.4	1.1	0.02	0.9	0.2
FA (kg)	0.1	0.0	0.1	0.0	0.04	0.0	0.03	0.01

Values are representing a normal batch ($n = 1$) of either TSp or SC.

Table 4COD (g/L), BOD₅ (g/L), TSS (g/L) and salt content (wt%) in RAW BRINE, PF-PER, UF-PER and UF-RET after consecutive PF and UF treatment of TSp and SC.

	TSp				SC			
	RAW BRINE	PF-PER	UF-PER	UF-RET	RAW BRINE	PF-PER	UF-PER	UF-RET
COD (g/L)	124 ± 2.5 ^{bc}	128 ± 2.4 ^{bc}	109 ± 2.5 ^c	121 ± 2.5 ^{ab}	151 ± 2.6 ^a	109 ± 0.4 ^c	87 ± 1.2 ^d	109 ± 0.4 ^c
BOD ₅ (g/L)	23 ± 0.3 ^a	23 ± 0.9 ^a	23 ± 0.3 ^a	23 ± 0.3 ^a	23 ± 0.2 ^a	24 ± 0.1 ^a	24 ± 0.0 ^a	23 ± 0.1 ^a
TSS (g/L)	16 ± 1.8 ^{ab}	8 ± 2.1 ^c	0.3 ± 0.1 ^d	7 ± 1.3 ^c	20 ± 3.5 ^a	16 ± 1.3 ^{ab}	1 ± 0.3 ^d	14 ± 3.8 ^{bc}
Salt (wt%)	13.4 ± 0.1 ^b	13.2 ± 0.1 ^{bc}	13.5 ± 0.2 ^{ab}	13.1 ± 0.1 ^{bcd}	13.8 ± 0.2 ^a	13.3 ± 0.2 ^b	12.8 ± 0.2 ^{cd}	12.8 ± 0.1 ^d

Values are given as mean (n = 2 for COD and BOD₅, n = 3 for TSS and salt) ± standard deviation (absolute value). By rows, same letter indicate no statistically difference (P < 0.05).

Table 5

Zn (μg/g), Fe (μg/g), Mg (μg/g), Ca (μg/g), P (mg/L) and N (mg/g), in RAW BRINE, UF-PER and UF-RET after consecutive PF and UF treatment of TSp and SC.

	TSp			SC		
	RAW BRINE	UF-PER	UF-RET	RAW BRINE	UF-PER	UF-RET
Zn (μg/g)	7.19 ± 0.176 ^a	6.06 ± 0.686 ^a	9.31 ± 1.50 ^a	6.89 ± 0.494 ^a	6.95 ± 0.629 ^a	6.36 ± 0.537 ^a
Fe (μg/g)	6.79 ± 0.339 ^a	0.61 ± 0.035 ^d	4.44 ± 0.057 ^b	2.38 ± 0.099 ^c	0.37 ± 0.148 ^d	1.73 ± 0.099 ^c
Mg (μg/g)	307 ± 0.813 ^b	277 ± 2.12 ^b	299 ± 2.68 ^b	533 ± 39.0 ^a	497 ± 20.0 ^a	495 ± 18.3 ^a
Ca (μg/g)	265 ± 8.83 ^a	160 ± 4.56 ^b	243 ± 7.52 ^a	133 ± 19.9 ^{bc}	160 ± 6.06 ^b	112 ± 7.02 ^c
P (mg/L)	2875 ± 5 ^c	3228 ± 19.0 ^b	3723 ± 73 ^a	1880 ± 36.1 ^e	2188 ± 11.5 ^d	1708 ± 38 ^f
N(mg/g)	14.4 ± 0.16 ^a	8.10 ± 0.01 ^d	13.8 ± 0.06 ^b	8.64 ± 0.04 ^c	3.50 ± 0.04 ^e	8.01 ± 0.00 ^d

Values are given as mean (n = 2 for Zn, Fe, Mg, Ca and N, n = 3 for P) ± standard deviation (absolute value). By rows, same letter indicate no statistically difference (P < 0.05).

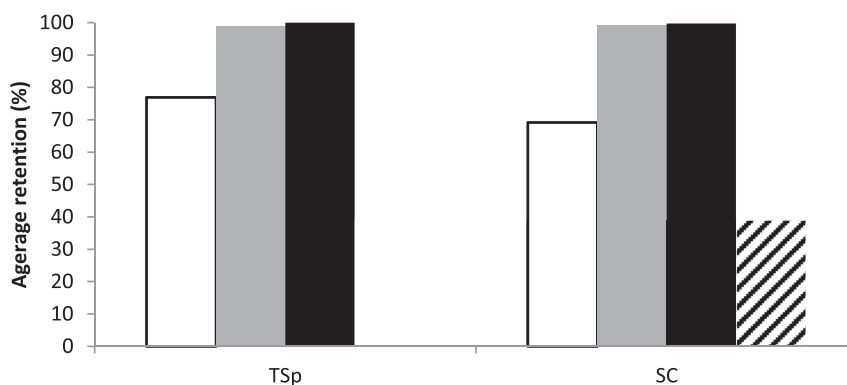


Fig. 2. Average retention of protein (white), total fatty acids (FA) (grey), total n-3-FA (black) and total phenolic compounds (striped) in TSp and SC after consecutive PF and UF treatment. The standard deviation is indicated by error bars, although all are less than 0.08.

mL in the UF-PER, i.e. the membranes had retained almost 40% phenolics. In TSp, no phenolics were retained and it is unclear what causes this difference between SC and TSp. It can be speculated that the phenolic compounds present in TSp are smaller in size due to a longer ripening time and might therefore pass through the membrane compared to the phenolics in SC which might be bigger and therefore retained. The retention of phenolics by ultrafiltration has been studied by Galanakis, Markouli, and Gekas (2013) with three different MWCOs (100, 20 and 1 kDa) and two different dilutions of winery sludge (containing 0.48 and 1.97 mg phenols/mL). The retention was 56–85%, which might be explained by the lower MWCO values used in their work compared to ours.

To determine if oxidation was exacerbated under UF conditions, TBARS-value of PERs and RETs were determined for TSp and SC. From Fig. 3 a notable difference between the two brines is seen which could be explained by the longer ripening time of TSp compared to SC. TSp RAW BRINE had 45 nmol MDAeq/mL, which decreased to 29 nmol MDAeq/mL in the UF-PER and increased to almost 70 nmol MDAeq/mL in the UF-RET. In contrast, SC RAW

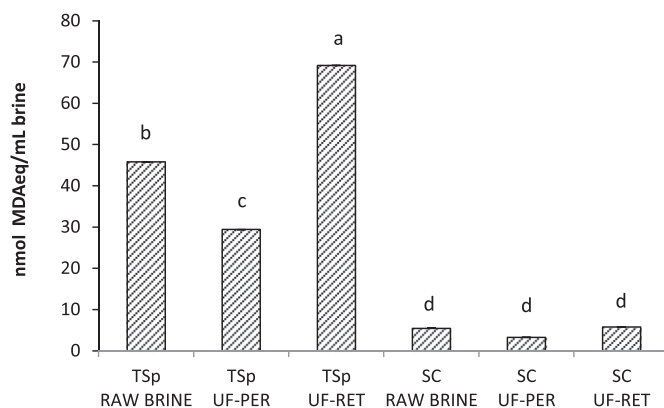


Fig. 3. Average TBARS (nmol MDAeq/mL brine) values in RAW BRINE, UF-PER and UF-RET of both TSp and SC (n = 3). The standard deviation is indicated by error bars, although all are less than 0.04. Letters indicate no statistically difference (P < 0.05).

BRINE had only 5.4 nmol MDAeq/mL which was unchanged in the UF-PER and UF-RET. These results indicate that, regarding oxidation, SC was stable during the PF and UF, whereas significant ($P < 0.05$) oxidation took place in the case of TSp, which was significantly ($P < 0.05$) more oxidized than the SC. The brines have different initial compositions and the retention of phenolics was different for the different brines, which may explain the observed results.

This study has tested the EF and PF in combination with UF for the recovery of high value biomolecules, i.e. protein, FA, n-3-FA and phenolics into retentates and permeates. The consecutive treatment with PF and UF reduced the COD, TSS, Fe and N content in the spice brines. The generated permeates were fat-free and contained a phenolic content of up to 3.8 mg GAE/mL, as well as a notable phosphorous content. In contrast, the retentates were loaded with proteins and lipids. This work indicates that ceramic UF can recover biomolecules from the spice brines generated during the marinated herring production although further optimization is needed in order to improve the separation of the HMW and LMW biomolecules.

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PAPER III

Gringer, N.; Safafar, H.; Mesnildot, A.d.; Nielsen, H.H.; Rogowska-Wrzesinska, A.; Undeland, I.; Baron, C.P. (2015). Antioxidative low molecular weight compounds in marinated herring (*Clupea harengus*) salt brine. *Food Chemistry*.

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Antioxidative low molecular weight compounds in marinated herring (*Clupea harengus*) salt brine

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Running title: Antioxidants in herring brine

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1 1. Introduction

2 In the final maturation step (also called ripening period) during production of
3 marinated herring, a salt brine which is rich in organic matter is generated. In a
4 former study this brine was shown to be rich in low molecular weight compounds
5 (LMWC) such as small proteins (e.g. in the form of enzymes), non-protein nitrogen,
6 iron and antioxidants (Gringer, Osman, Nielsen, Undeland and Baron, 2014). The
7 antioxidant activity present in the salt brine was revealed to be related to the
8 processing methods. Indeed, ripening of headed herring generated brines with
9 higher antioxidant activities compared to herring processed as fillets. This might be
10 due to the intestinal proteases present in the brine from the headed herring, which
11 may generate lower molecular weight peptides with antioxidant activity. Further, our
12 recent work (Gringer, Hosseini, Svendsen, Undeland, Christensen and Baron, 2015)
13 showed that brines from spice-cured marinated herring, both in a headed and filleted
14 form, contained high amounts of phenolic compounds, and it is thus possible that the
15 documented antioxidant activity in these brines is a result of phenolics released from
16 the spices (Shahidi and Zhong, 2010, Gil-Chávez et al., 2013). It could, however,
17 also be a combination of both the phenolic compounds originating from spices and/or
18 the presence of peptides/free amino acids as a result of proteolysis.

19 Certain peptides, usually 2-20 amino acid residues in length, can exhibit a range of
20 bioactivities; the activity being dependent on the sequence of amino acids. For
21 example, peptides originating from foodstuff with immunomodulatory, antimicrobial,
22 antihypertensive, antioxidant and even multifunctional actions have been described
23 by Hartmann and Meisel (2007). Fish peptides with for example the tripeptides LKP,
24 IKP and LRP originating from fish muscle have been shown to have antihypertensive
25 effects (Nagai, Suzuki and Nagashima, 2006) and the dipeptide MY from sardine

muscle has been shown to be antioxidative (Erdmann, Grosser, Schipporeit and Schöder, 2006).

Within the food industry, there is an increasing focus on recovery of natural antioxidants in order to replace some synthetic ones. Therefore, the recovery of LMWC with antioxidant activity such as peptides and plant phenolics is an important step towards the replacement of synthetic antioxidants. An example is the recovery of phenolics from olive mill wastewater (Rahmanian, Jafari and Galanakis, 2014). The interest in the use of natural antioxidants in the food industry stems not only in the applicability as preservatives but also in their tentative benefits to human health (Shahidi and Zhong, 2010, Barbosa-Pereira, Pocheville, Angulo, Paseiro-Losada and Cruz, 2013). Thus, both novel sources of antioxidants e.g. from food processing waste and new purification processes are needed (Gil-Chávez et al., 2013). However, despite the increasing interest in natural antioxidants, to the best of our knowledge no previous study have evaluated and characterised the antioxidants present in the salt brines from the marinated herring industry.

The objective of the present work was to characterise the antioxidants present in different salt brines from traditional headed barrel-salted herring; with spices (TSp) and without spices (TSa), as well as from fillet-ripened spice-cured herring (SC). The antioxidant activity was linked to specific peptides and phenolic compounds that were further characterised.

46

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from Merck (Darmstadt, Germany), Fluka (Deisenhofen, Germany) or Sigma-Aldrich (Steinheim, Germany) and were of

51 analytical grade. Water used throughout the study was double deionized water
 52 (dH₂O), or High Performance Liquid Chromatography grade water for the HPLC
 53 analysis.

54

55 2.2. Herring brine samples

56 2.2.1. Raw brines

57 Brines from the last maturation steps in the production of marinated herring (*Clupea*
 58 *harengus*) were obtained from Lykkeberg A/S (Hørve, Denmark), and were prepared
 59 according to Lykkeberg A/S production protocols from herring caught in the North
 60 Atlantic. Three brines were obtained; TSa, TSp and SC. TSa and TSp both resulted
 61 from salting whole herring (200 days), filleting and then placing the fillets back in the
 62 brine for additional ripening (approx. one year), whereas SC resulted from ripening of
 63 fillets for approx. 190 days. Besides salt, TSp and SC contained a considerable
 64 amount of sugar and spices (undisclosed levels); whereas TSa only contain salt.
 65 Brines samples were stored at -80°C until further analysis.

66 All compositional analyses of the raw (untreated) brines were measured on a
 67 supernatant resulting from centrifugation at 13684 g, 3 min (Biofuge® Pico, Kendro,
 68 UK).

69

70 2.2.2. Fractionation of crude brines

71 A <10 kDa fraction of the brines was collected by submersion of 30 cm dialysis
 72 membrane (Spectra/Por® 6, MWCO 10,000 Da, Spectrum Lab. Inc., CA, USA)
 73 containing 50 mL (corresponding to 50% membrane volume) dH₂O into 2 L of the
 74 raw brine. After 22 h (at room temperature on a magnetic stirrer), 22-25 mL <10 kDa
 75 fractions were collected from the membrane and stored at -20°C until analysis.

Size exclusion chromatography (SEC) was performed on the three <10 kDa fractions using fast performance liquid chromatography (FPLC) equipment (Äkta Purifier system with Frac 950 collector) according to Falkenberg, Mikalsen, Joensen, Stagsted and Nielsen (2014) with a few modifications. Briefly, 50 µL of the <10 kDa fraction was injected into a SuperdexTM peptide 10/300 GL column (GE Healthcare), using a 100 mM ammonium acetate buffer at pH 8 as running buffer and a flow rate of 0.25 mL/min. Eluting compounds were detected at 215 and 280 nm, respectively. Cytocrome c (12.3 kDa), aprotinin (6.5 kDa), Gly₃ (189 Da) and Gly (75 Da) (Sigma-Aldrich, St. Louis, Missouri, USA) were used as molecular weight markers. For each <10 kDa fraction, 94 fractions of 320 µL each were collected into a 96 well microplate and samples were stored at -20°C until further analysis. Based on the enrichment of proteins in the fractions eluting after 18-19.5, 21.5-23, and 28-29.5 mL (see Results section), these fractions were pooled into P1, P2 and P3, respectively, and were subjected for deeper characterisations.

In the case of <10 kDa fractions, fractions resulting from FPLC, and the pooled fractions P1-P3, no pre-centrifugation of the samples was carried out prior to compositional analyses.

2.3. Analytical methods

2.3.1. Proximate composition

pH was measured directly in the brine using a Metrohm 827 pH meter (Herisau, Switzerland). The NaCl content (w/w %) was measured according to the AOAC standard method (AOAC, 2005). Dry matter (DM) content was measured by a two-step evaporation of water at 60°C for 24 h followed by 105°C for 24 h. The DM (%) was calculated as (mass of dry sample * 100)/(mass of wet sample). Ash content (%)

was measured by combustion at 600°C for 24 h, and is reported as (mass of ash * 100)/(mass of wet sample). The protein content (mg/mL) was obtained using the bicinchoninic acid (BCA) kit (Thermo Scientific, Pierce®, Rockford, USA) with bovine serum albumin (BSA) as standard, and detected spectrophotometrically at 562 nm (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instruments, Inc., Vermont, USA).

2.3.2. Antioxidant assays

Iron (II) chelating activity was assayed in raw brines, <10 kDa fractions and the 94 SEC-generated fractions by the method described by Farvin, Baron, Nielsen, Otte and Jacobsen (2010), reducing power was measured according to the protocol described by Oyaizu (1986), and ABTS-radical scavenging activity was determined according to the method described by Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999). All three assays are performed as described in Gringer et al. (2014), with volumes adjusted to fit microplate detection. All assays were based on spectrophotometric detection using a microplate reader (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instruments, Inc., Vermont, USA), with assay controls (dH₂O or ammonium acetate buffer instead of sample) and a sample control (dH₂O instead of reagents). The positive controls were ethylenediaminetetraacetic acid (EDTA, 100 mM), ascorbic acid (0.5 mM) and Trolox (2.5 mM, in 96% ethanol) for iron chelating, reducing power and ABTS-radical scavenging assays, respectively. Iron chelating activity was measured at 562 nm and reported as %-activity compared to the dH₂O (0% activity). The reducing power was measured following 10x dilution of samples at 700 nm and was expressed as OD₇₀₀; increasing absorbance indicates

increased reducing power. The ABTS-radical scavenging activity was expressed as OD₇₃₄ and a negative value indicates a high radical scavenging activity.

2.3.3. Total phenolic content and identification of phenolic acids

Total phenolic content (TPC) was determined in raw brines, <10 kDa fractions and the 94 SEC-generated fractions according to Singleton and Rossi (1965). Briefly, 20 μ L of sample was mixed with 150 μ L 10% Folin-Ciocalteu reagents and incubated at room temperature for 10 min. Then 150 μ L 6% sodium bicarbonate was added and the mixture was incubated at room temperature for 90 min in the dark. Subsequently, the samples were vortexed and centrifuged (3 min at 13684 g) before the supernatant (200 μ L) was transferred to a microplate and the absorbance was read at 725 nm. A calibration curve was prepared with gallic acid (GA) and TPC is reported as mg gallic acid equivalents (GAE) per ml brine:

$$\frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) - \text{intersect of calibration curve}}{\text{slope of calibration curve}} \cdot \text{dilution factor}$$

The identification and quantification of specific phenolic acids were determined in the 9 pooled fractions (P1-P3 of three brines) by HPLC. Aliquots of 0.1 mL of each sample were diluted in 1 mL of methanol and incubated at 37°C for 30 min in a rotary shaker, to recover the phenolic acids. The suspension was centrifuged for 15 min at 1164 g to remove e.g. salt and the supernatant (methanol/water-phase with phenolic acids) was recovered and used for analysis. Extracts were filtered using 0.22 μ m polyvinylidene defluoride (PVDF) syringe filter, and then analysed by HPLC using an Agilent 1100 Liquid Chromatograph (injection volume was 20 μ L) equipped with a diode array detector (DAD). The separation was carried out in a Prodigy ODS-3 column 250 mm x 46 mm with 5 μ m particle size from Phenomenex (Torrance,

USA). The mobile phase was a mixture of solvent A (phosphoric acid in deionised water, pH=3) and solvent B (acetonitrile) at 0.9 mL/min. The gradient started with 95:5 (A:B) and after 2 min increased to 60:40 (A:B) in 20 min and a subsequent increase to 100% B at 15 minutes and finally kept constant for 25 minutes. Total acquisition time was 70 minutes. The identification of the peaks was done using 4-hydroxybenzoic acid, salicylic acid, cinnamic acid, 2,5-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, p-coumaric acid, 4-hydroxy-3-methoxybenzoic acid, gallic acid, caffeic acid, trans-4-hydroxy-3-methoxycinnamic acid, syringic acid, (+)-catechin hydrate, (-)-epigallocatechin, chlorogenic acid, (-)-epicatechin gallate and (-)-epigallocatechin gallate as standards.

2.3.4. Total amino acids

Total amino acids were analysed in the 9 pooled samples according to the method described by Farvin et al. (2010). In short, 100 µL samples were hydrolysed for 1 h in a microwave (Multiwave 3000, Anton Paar, Austria), at 110°C at 500 W, in 400 µL dH₂O + 500 µL of 12 M HCl in sealed ampules. The samples were diluted (x3) in NaCO₃ and filtered through a 0.2 µm membrane filter before derivatisation using the EZ: Fast kit from Phenomenex A/S (Allerød, Denmark). 25 µL of the sample were injected into HPLC fitted with the reversed phase column EZ: Fast AAA-MS (250 x 3.0 mm, Phenomenex A/S, Allerød, Denmark), and eluted at 35°C with a flow rate of 0.5 mL/min. Mobile phase A (water) and B (methanol) both contained 10 mM ammonium formate, and the initial ratio of A:B was 40:60. The programme consisted of a linear increase to 83% B in 20 min, and a subsequent re-equilibration of the column to 60% B (6 min). The eluate was transferred to the on-line mass spectrophotometer (MS) (Aligent 1100, Aligent Technology, Waldbronn, Germany)

where amino acids were ionized using atmospheric pressure chemical ionization (APCI) with scanning from 100 to 600 m/z. The amino acids were quantified based on peak areas of internal standards. The total amino acid content is expressed in $\mu\text{g/mL}$ brine. This procedure did not allow the detection of methionine, tryptophan and cysteine due to the use of HCl during the hydrolysis.

2.3.5. Qualitative analysis of peptides and proteins

Direct peptide analysis was carried out in the 9 pooled samples after desalting and up-concentration using in-house made micro-columns packed with POROS 20R2 RP (Applied Biosystems, USA) material as described previously by Gobom, Nordhoff, Mirgorodskaya, Ekman and Roepstorff (1999). Briefly, vacuum-dried samples were resuspended in 20 μL 3.5% trifluoroacetic acid (TFA) and loaded onto the R2 micro-column. Washing with 10 μL of 1% TFA was followed by elution with 3 μL of matrix solution (5 $\mu\text{g/mL}$ α -cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.1% TFA) directly onto the MALDI target (Opti-TOF 384 Well Insert, Applied Biosystems).

MS and MS/MS spectra were acquired in positive reflector ion mode on a 4800 Plus MALDI TOF-TOF instrument (Applied Biosystems). Due to the very limited sample amount, the majority of the MS scans (m/z range of 700–3500) were acquired manually and the spectra were accumulated until sufficient intensity was achieved (on average 2500–4000 laser shots per spot). Spectra were calibrated externally to a standard β -lactoglobulin tryptic digest. Collision-induced dissociation of manually selected precursors was performed automatically with default calibration.

P2 and P3 (see Results section) contained peptides of unknown sizes, thus were analysed for intact small proteins. The method was similar with the direct peptide analysed above, with the exception of POROS 50R1 (Applied Biosystems) material

that was used to enhance retention of small proteins. Matrix solution used for elution of proteins was 5 μ g/L α -cyano-4-hydroxycinnamic acid in 70% ACN, 0.1% TFA. To identify the origin of the detected small proteins found in P2 and P3 fractions, samples were digested using a trypsin solution (6 ng/L trypsin in 50 mM ammonium bicarbonate) for 18 hours at 37°C (15–20 μ L). The resulting peptides were desalted and analysed by MALDI MS as described above. MS and MS/MS spectra were analysed manually.

2.4. Statistical analysis

Measurements were carried out in triplicates unless otherwise stated. Results are given as mean values \pm absolute standard deviations. For all statistical analysis, the GraphPad Prism® software Ver. 4.03 was used with $P < 0.05$. Results were compared using one-way ANOVA test with Tukey's post-test.

3. Results and discussion

3.1. Characterisation of raw brines and their <10 kDa fractions

3.1.1. Proximate composition

The proximate composition and the antioxidant activity of three raw brines (TSa, TSp and SC) and their <10 kDa fractions is given in Table 1. For all three brines, a significant ($P < 0.05$) lower protein concentration was observed in the <10 kDa fraction, which indicate that the majority of the proteins in the brines are high molecular weight (HMW) proteins. The raw SC had a protein concentration of 32.51 mg/mL which was reduced to 3.04 mg/mL after dialysis, and the raw TSa had 44.37 mg/mL protein with after dialysis reached only 4.25 mg/mL. For both SC and TSa the protein concentration reached approx. 10% of the initial protein concentration. The

raw TSp protein concentration was reduced from 41.48 to 9.47 mg/mL following dialysis, corresponding to approx. 23% of the initial. Taken into consideration the different volumes of the raw brines versus that of the dialysed fractions (2L vs. 22-25 mL), 0.11% of the protein in SC and TSa were detected in the dialysate, whereas 0.26% in the case of TSp; Thus, this indicates that SC and TSa contained significantly ($P < 0.05$) less low molecular weight protein/peptides (< 10 kDa) compared to TSp. As TSp and TSa both are prepared from headed herring, were matured for equally long times and had identical initial protein concentrations, it could be expected that these two brines would have a similar protein contents in the < 10 kDa fractions. The reason for this difference might be due to the presence of sugar in TSp, as sugar has been shown to enhance protease activity (Rangaswamy, Nagaraju and Narasimha, 2009), which in term could have promoted the degradation of the protein to smaller peptides (< 10 kDa). In contrast, interaction between some (poly)phenols and protein is known to lead to protein-precipitation (Arts, Haenen, Voss and Bast, 2001), which may affect TSp resulting in less protein/peptides in the < 10 kDa fraction of TSp, as this dialysate had the highest phenolic content.

Many spices, which are commonly added to the brine in the preparation of marinated herring, are known to contain phenolic compounds, e.g. rosemary, sage, oregano, thyme, and black pepper (Madsen and Bertelsen, 1995, Yanishlieva, Marinova and Pokorný, 2006). Thus, the concentration of TPC in the three raw brines and the < 10 kDa fractions were analysed (Table 1). For TSa no significant ($P < 0.05$) change was observed from raw brine (0.16 mg/mL) to < 10 kDa fraction (0.14 mg/mL), whereas the TPC of SC was significantly ($P < 0.05$) decreased (0.26 to 0.17 mg/mL) in the < 10 kDa fraction compared to the raw brine. In contrast to SC, TPC of TSp was

significantly ($P < 0.05$) higher in the <10 kDa fraction (0.26 mg/mL) compared to the raw brine (0.16 mg/mL). These results imply that although raw SC brine contained the highest TPC-level, a large part of these compounds are larger than 10 kDa. The opposite seemed to be true for TSp, i.e. the majority of the TPC were smaller compounds perfectly capable of penetrating the 10 kDa dialysis membrane resulting in a higher concentration of TPC in the <10 kDa fraction compared to the raw brine.

The higher level of TPC present in the spice brines (TSp and SC, identical levels of spices) correspond well with the contribution from the spices when compared to TSa. However, a relatively high TPC was found in TSa which showed that phenolic compounds others than the one originating from spices (probably originating from peptides) are contributing to the TPC. The proteolytic activity present in TSp (Gringer et al., 2014) may explain why TPC is higher in the <10 kDa TSp compared to SC, with proteolysis in the former leading to small peptides and amino acid contributing to TPC.

In order to evaluate the relationship between protein/peptides (P) and TPC, the concentration of TPC was expressed as the ratio TPC/P (see Table 1). SC had the highest TPC/P ratio with 8.0 mg/g, whereas raw TSp and TSa had similar initial ratio of 3.9 and 3.6 mg/g, respectively. In the <10 kDa fractions, SC had almost twice the amount of TPC/P compared to TSp and TSa; 55.9 mg/g vs. 27.5 mg/g and 32.9 mg/g, respectively. This implies that even though the protein content in SC was in general lower than in the two traditional barrel-salted brines, the spice-derived phenolics in SC made up the larger part of the TPC compared to that in TSp and TSa. Moreover, as raw TSp and TSa brines had the same level of phenolics this

further support the fact that the TPC in these brines are not solely due to the spices, but also to preservatives, peptides or free aromatic amino acids.

3.1.2. Antioxidant activity

Raw brines and their respective <10 kDa fractions were analysed for their antioxidant activity; ABTS-radical scavenging, reducing power and iron chelating activity, as reported in Table 1. TSp had the strongest ABTS-radical scavenging activity; however the activity in the <10 kDa TSp was slightly lower than in the raw brine. The same tendency was observed for TSa; however significantly ($P<0.05$) lower activities were seen in TSa-samples compared to TSp. For SC the ABTS-radical scavenging activity in <10 kDa fraction was significantly ($P<0.05$) higher than in the raw brine, and at the same level as for the <10 kDa fractions of TSa and TSp. Radical scavenging activity of peptides is most likely due to the phenolic and indolic groups (Pihlanto, 2006) and whether the ABTS-radical scavenging activity found in the three <10 kDa fractions was solely due to the phenolic and indolic groups originating from spices or from peptides/free amino acids is unclear.

The reducing power decreased significantly ($P<0.05$) in all three <10 kDa fractions compared to the initial raw brines (0.39 to 0.12 for SC; 0.69 to 0.28 for TSp; 0.69 to 0.18 for TSa). The TSp <10 kDa fraction retained some reducing power when compared to TSa and SC, which could be due to the higher protein content in the <10 kDa TSp fraction. Both the raw TSp and TSa brines had almost the same reducing power as ascorbic acid (0.5 mM). Taheri, Farvin, Jacobsen and Baron (2014) measured the reducing power in a 1-10 kDa fraction of salt brine and found that it correlated well with protein content. This is in line with our data, as a lower reducing power was found in the <10 kDa fractions compared to the more protein

loaded raw brines. The iron chelating activity did not seem to correlate with either TPC or protein. All the raw brines had an activity of 81-93%, whereas the <10 kDa fractions had an activity of 55.33%, 58.51% and 92.19% for SC, TSa, and TSp, respectively. Thus, raw brine and <10 kDa fraction of TSp brine were excellent iron chelators. It is possible that the high iron chelating activity in the <10 kDa TSp fraction was linked to the high TPC-content of this fraction (0.26 mg/mL); many phenolics are known to strongly chelate metals (Andjelkovic et al., 2006). These results are in clear contrast to the iron chelating activity, of the 1-10 kDa fraction of TSa, reported by Taheri et al. (2014), as they only found negligible chelating activity. On the other hand, Torres-Fuentes, Alaiz and Vioque (2012) studied iron chelating activity in a protein hydrolysate from chickpeas and found that in general, fractions <500 Da had higher iron chelating activities than the fractions >500 Da. This could point to the fact that the high activity found in our samples are due to very low molecular size molecules (<500 Da) which were not present in the 1-10 kDa fraction studies by Taheri et al. (2014). Whether the iron chelating activity was a result of the peptides or the phenolics (measured as TPC) is questionable. However, Rice-Evans, Miller and Paganga (1996) have claimed that metal chelation plays a minor role in the overall antioxidant activity of phenolics, and hence this activity may be solely related to the protein/peptide content.

Data show that the processing method, traditional barrel-salting of headed herring versus ripening of filleted herring, had a major impact on the composition and the antioxidant activity of the brine and of the <10 kDa fractions with the LMWC. The addition of spices also had a notable impact on the TPC, and the combination of the traditional marinating method and the use of spices seem to improve the antioxidant profile of the LMWC in the brine.

325

326 3.2. Characterisation of the fraction obtained by size exclusion chromatography 327 (SEC)

328 In order to study the LMWC more in detail, the three <10 kDa samples were further
329 fractionated by SEC which resulted in the chromatograms in Figure 1. Similarities
330 between TSp and TSa were revealed with respect to peptides and aromatic rings,
331 which contrasted to the chromatogram obtained for SC which was giving less intense
332 peaks at both 215 and 280 nm. This point to the fact that processing methods (whole
333 herring vs. fillets), rather than the addition of spices, are more profoundly affecting
334 the relative level of protein/peptides in herring brines. Indeed, Nielsen (1995) has
335 earlier reported the profound effect of proteolytic enzymes from herring viscera on
336 the ripening of traditional barrel-salted herring. The difference between marinating
337 with or without spices (i.e. SC and TSp vs. TSa) was expected to be seen in the
338 chromatogram at 280 nm (aromatic rings), but this was not the case as TSa seemed
339 to have more phenolic compounds compared to TSp and this cannot be fully
340 explained.

341 The SEC resulted in a total of 94 fractions that eluted after 8-38 mL which were
342 analysed for protein concentration and TPC, and tested for their antioxidant activities
343 (Figure 2). Figure 2A showed three main protein peaks P1, P2 and P3 corresponding
344 to the fractions eluting after 18-19.5, 21.5-23, and 28-29.5 mL respectively. The high
345 protein content detected in TSp <10 kDa fraction was undoubtedly eluting in P1,
346 which corresponds to peptides of the size equal to Gly-Gly₃ (75-189 Da). In
347 agreement with previous observations, TSp-P1 had more pronounced reducing
348 power (Figure 2D). P1 peaks had a minor amount of phenolics (Figure 2B), ABTS-
349 radical scavenging activity (Figure 2C) and iron chelating activity (Figure 2E). The P2

peaks were eluting after the water from the samples eluted, thus these peptides were retained on the column due to hydrophobic interaction and it was consequently not possible to determine their molecular size. Nevertheless, these protein peaks contained the greatest part of the phenolics (Figure 2B), and demonstrated a strong ABTS-radical scavenging (Figure 2C), and almost no reducing power (Figure 2D) and iron chelating activity (Figure 2E). The third protein peak, P3s (also eluted after water), represented less protein and phenolics than P2s (Figure 2B) but hosted a significant ABTS-radical scavenging activity (Figure 2C). Considering our data (Figure 2A-C), the ABTS-radical scavenging activity seemed to be mainly linked to the TPC. The iron chelating activity (Figure 2E) was increasing in the fraction eluting after 8 to 16.5 mL, for TSp and TSa, and from 8 to 18 mL in the case of SC. The activity then remained insignificant after 22 mL. The results from the iron chelating activity indicated that this activity may be attributed to small peptide as also reported by Andjelkovic et al. (2006). However, it has been reported that smaller peptides are less able to chelate metals than larger peptides (Khantaphant, Benjakul and Kirhimura, 2011). Earlier investigations showed that salt brine do contain antioxidant activity (Gringer et al., 2014), and here we identify that the antioxidative activity is present in the <10 kDa fraction. In agreement with our findings, Pampanin, Larssen, Provan, Sivertsvik, Ruoff and Sydnes (2012) have reported, that the antioxidative properties in extracts from herring skin and filleting cut-offs were attributed to small peptides, <10 kDa. In contrast, Sannaveerappa, Carlsson, Sandberg and Undeland (2007) reported that the antioxidant activity of herring muscle press juice was mainly caused by small organic acids such as ascorbic acid and uric acid, and thus more detailed information about the nature of the herring brine antioxidants is needed.

375

376 3.3. Identification of low molecular weight compounds (LMWC)

377 3.3.1. Phenolic acids

378 The three pooled samples, P1, P2 and P3, (Figure 2), were collected for further
379 identification of phenolic acids, total amino acids and peptides. The data presented
380 in Table 2 shows that these pooled fractions only contained five out of the 16
381 common phenolic acids tested for, in addition to several unidentified compounds.
382 The unidentified peaks might correspond to more complex phenolic compounds.
383 None of the P1s contained any of the phenolic acids analysed for, which correlates
384 well with data from the TPC assay (Figure 2B). Gallic acid was present in all P2s and
385 was the only measurable phenolic acid in SC-P2 and TSa-P2 with values of 5.63 and
386 5.70 $\mu\text{g/mL}$, respectively. In TSp-P2 the concentration of gallic acid was higher, 8.96
387 $\mu\text{g/mL}$, and this fraction also contained caffeic acid, vanillic acid and ferulic acid at
388 concentrations of 1.60, 35.32 and 65.33 $\mu\text{g/mL}$, respectively. P3 fractions all
389 contained 2,5-dihydroxybenzoic acid, however at very low concentrations; 0.32, 0.27
390 and 1.06 $\mu\text{g/mL}$ for SC, TSp and TSa, respectively. Of the three P3s, only TSp
391 contained other phenolic acids, i.e. caffeic acid and vanillic acid. From Table 2 there
392 is no doubt that TSp is the brine with most phenolic acids, supporting that the highest
393 TPC level was observed in the <10 kDa TSp fraction, in agreement with previously
394 reported data (Table 1). However, in this work non-phenolic acid phenols and
395 polyphenols were not identified and these could also contribute to the antioxidant
396 activity present in the brine. Indeed a study on herring muscle press juice reported
397 that small organic acids and LMWC had a strong antioxidative activity towards
398 hemoglobin-mediated oxidation of washed cod mince (Sannaveerappa et al., 2007).

There are several studies describing the identification and quantification of phenolic compounds in the brine and processing water generated in table-olive oil production (e.g. Bouaziz et al., 2008). However, recovering of phenolics from marinades and salt brines could represent a new source of natural antioxidant. Indeed, we demonstrate here for the first time that there is a non-negligible level of phenolic acids in marinated herring brines that may be recovered.

3.3.2. Total Amino acids

The total amino acid composition shows a clear difference between P1, P2 and P3 for all three brines. Table 3 shows some amino acids with known antioxidant activity. Phenylalanine was detected in TSp-P1 and -P2 in levels of 13 and 31 $\mu\text{g/mL}$, respectively. The two barrel-salted versions show proline in P1 and tyrosine in P2, whereas histidine was not detected in any of the pooled samples. In general, P1s contained a variety of amino acids, and as expected, the two barrel-salted brines had higher total amino acid load compared to SC. In a previous study (Gringer et al., 2014) we demonstrated that raw brines were rich in lysine, alanine, aspartic acid and asparagine, glutamic acid and glutamine, glycine and serine. Despite the very complex amino acids profile of the raw brines used in this experimental set up, only TSp-P1 contained alanine, and glycine was not detected in any of the pooled samples (data not shown). Such differences cannot be explained, but could be due to batch variation.

3.3.3. Protein and peptides

As both P2 and P3 fractions eluted after the water, their size could not be directly determined from the chromatograms, and P2s and P3s were analysed by MALDI

MS. The results indicated the presence of a small protein fragment in both P2 and P3 with m/z 8604 Da (data not shown). In an attempt to determine the identity of the protein fragment above, trypsin digestion of the P2/P3 samples together with MALDI MS and MS/MS analysis of the trypsin digest obtained was performed. Digested TSp-P2 contained two peptides (1567.7 and 2134.0 Da) and TSa-P2 contained four peptides (1099.5, 1481.7, 1543.7 and 1559.7 Da). Unfortunately, the MS/MS fragmentation of the observed peptide peaks was very poor and did not give sufficient fragment ions to determine the peptides' amino acid sequence. This might be due to the fact that the protein fragments were not digestible by trypsin due to modification of the amino acids lysine and arginine induced by the complex brine. In parallel, MS analysis of peptides was carried out in P1, P2 and P3 from all three brine samples. Purified (non-digested) samples were directly spotted on MALDI target and subjected to MS and MS/MS analysis. TSp-P1 analyses revealed the presence of two peptides for which *de-novo* sequencing revealed fragments of the sequence (see Table 4). Interestingly, SC contained the highest number of detectable peptides. For 4 out of the 6 peptides observed, peptide sequence information revealed that they had the same C-terminal sequence. At the same time the mass differences in the intact mass of the peptides suggests that they differ in the N-terminal sequence composition. Unfortunately the longest sequence tag obtained spans only 6 amino acids (VY[L/I]HDF) and was not sufficient for unambiguous identification of the initial protein/peptide it is stemming from. Difficulties in the manual interpretation of the peptide fragmentation spectra also suggested that the amino acid residues in peptides present in SC-P3 (HDF) might be modified, but unfortunately, we were not able to determine the nature of the modification.

A relationship between antioxidant properties of peptides and the presence of aromatic (tryptophan, tyrosine and phenylalanine), imidazole (histidine) and sulphur-containing (cysteine and methionine) amino and imino acids has been shown (Shahidi and Zhong, 2010). However, as observed with the MS analysis, the peptides detected in P1s did contain amino acids that have documented antioxidant capacity i.e. phenylalanine, proline and tyrosine.

From Table 3 it is seen, that SC-P2 and SC-P3 did not contain any amino acids even if the MS revealed the presence of some small peptides (Table 4). This might be due to the fact that the method for total amino acid is far less sensitive compared to the MS analysis. This, together with the results from Table 3 and 4, supports that the ABTS-radical scavenging activity may be caused by phenolic groups from protein residues such as tyrosine and phenylalanine, whereas both reducing power and iron chelating activity seems to be solely linked to the proteinous fraction.

4. Conclusion

This study demonstrate that the LMWC (<10 kDa) in herring salt brines is composed of phenolic compounds and protein/peptides which are a good source of antioxidants. From the MS and MS/MS analysis, the motif HDF was found in the majority of the detected peptide sequences, and could be responsible for some of the antioxidant activity measured in the brines. We show here, that the addition of spices during the herring processing has some effect on the antioxidative character of the LMWC, especially on the ABTS-radical scavenging activity. In contrast, the peptides seem to be responsible for the iron chelating activity and the reducing power. However, more studies are needed to obtain more specific knowledge about

the contribution from the spices and the peptides, and their interaction, to the antioxidant activity in herring salt brine.

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604

605 **Figure 1** Size exclusion chromatograms at 215 nm (peptides) and 280 nm (aromatic
606 rings) of <10 kDa fractions from SC (spice-cured fillets), TSp (barrel-salted spice-
607 cured) and TSa (barrel-salted). Peptide standards are shown in the top figure
608 (Cytochrome C, 12.3 kDa; Aprotinin, 6.5 kDa; Gly₃, 189 Da and Gly, 75 Da), and the
609 elution of water from the samples is indicated with an arrow.

610

611 **Figure 2** Characterisation of the 94 SCE fractions emerging from fractionation of SC
612 (spice-cured fillets), TSp (barrel-salted spice-cured) and TSa (barrel-salted). A)
613 protein content (mg/mL), B) total phenolic compounds (TPC) (mg/mL), C) ABTS-
614 radical scavenging (OD₇₃₄), D) reducing power (OD₇₀₀), and E) iron chelating activity
615 (%). The three pooled fractions (P1, P2 and P3) are indicated in A) and the peptide
616 standards are shown (Cytochrome C, 12.3 kDa; Aprotinin, 6.5 kDa; Gly₃, 189 Da and
617 Gly, 75 Da) in the B). All standard deviations are below 10%.

618 e detected in the representative MS spectrum.

Table 1 pH, dry matter (%), ash (%), salt (%), protein (mg/mL) content, total phenolic compounds (TPC) (mg/mL), TPC/protein (TPC/P) ratio (mg/g), ABTS-radical scavenging (OD₇₃₄), reducing power (OD₇₀₀), and iron chelating activity (%) in the raw brines of SC (spice-cured fillets), TSp (barrel-salted spice-cured) and TSa (barrel-salted) and their corresponding <10 kDa fractions.

Brine sample	SC		TSp		TSa	
	Raw brine	<10 kDa	Raw brine	<10 kDa	Raw brine	<10 kDa
pH (-)	5.52 ± 0.04 ^d	5.81 ± 0.08 ^{b,c}	5.78 ± 0.06 ^c	5.83 ± 0.02 ^{b,c}	5.93 ± 0.03 ^{a,b}	6.00 ± 0.03 ^a
Dry matter (%)	25.85 ± 0.18 ^a	20.31 ± 0.09 ^b	24.46 ± 1.31 ^a	19.98 ± 1.12 ^b	25.18 ± 0.09 ^a	21.17 ± 0.02 ^b
Ash (%)	15.67 ± 0.03 ^b	15.62 ± 0.01 ^b	13.62 ± 0.48 ^c	13.59 ± 0.72 ^c	17.46 ± 0.02 ^a	17.70 ± 0.02 ^a
Salt (%)	15.44 ± 0.10 ^b	15.30 ± 0.23 ^{b,c}	12.55 ± 1.08 ^d	13.63 ± 0.98 ^{c,d}	15.97 ± 0.52 ^{a,b}	17.54 ± 0.14 ^a
Protein (mg/mL)	32.51 ± 2.01 ^c	3.04 ± 0.01 ^e	41.48 ± 1.02 ^b	9.47 ± 0.87 ^d	44.37 ± 1.76 ^a	4.25 ± 0.09 ^e
TPC (mg/mL)	0.26 ± 0.012 ^a	0.17 ± 0.014 ^b	0.16 ± 0.013 ^b	0.26 ± 0.006 ^a	0.16 ± 0.024 ^b	0.14 ± 0.021 ^b
TPC/P (mg/g)	8.0	55.9	3.9	27.5	3.6	32.9
Radical scavenging (OD ₇₃₄)	-0.22 ± 0.014 ^a	-0.29 ± 0.002 ^b	-0.42 ± 0.009 ^d	-0.34 ± 0.005 ^c	-0.34 ± 0.019 ^c	-0.27 ± 0.006 ^a
Reducing Power (OD ₇₀₀)	0.39 ± 0.026 ^b	0.12 ± 0.008 ^d	0.69 ± 0.066 ^a	0.28 ± 0.019 ^{b,c}	0.69 ± 0.114 ^a	0.18 ± 0.011 ^{c,d}
Iron chelating activity (%)	93.42 ± 1.29 ^a	55.33 ± 6.36 ^c	81.01 ± 11.47 ^{a,b}	92.19 ± 2.80 ^a	87.24 ± 9.79 ^a	58.51 ± 11.12 ^{b,c}

Values are given as mean (n = 3) ± standard deviation (absolute value). In each row, same letters indicate homogeneous values (P<0.05).

Table 2 The major phenolic acids ($\mu\text{g/mL}$ sample) in the P1-P3 fractions emerging from SCE-separation of <10 kDa fractions from SC (spice-cured fillets), TSp (barrel-salted spice-cured) and TSa (barrel-salted).

Phenolic acids	SC			TSp			TSa		
	P1	P2	P3	P1	P2	P3	P1	P2	P3
Gallic	-	5.63 ± 0.035	-	-	8.96 ± 2.54	-	-	5.70 ± 0.028	-
2,5-Dihydroxybenzoic	-	-	0.32 ± 0.003	-	-	0.27 ± 0.007	-	-	1.06 ± 0.007
Caffeic	-	-	-	-	1.60 ± 0.127	1.35 ± 0.457	-	-	-
Vanillic	-	-	-	-	35.32 ± 1.90	35.04 ± 0.557	-	-	-
Ferulic	-	-	-	-	65.33 ± 2.36	-	-	-	-
Total	0	5.63	0.32	0	111.21	36.66	0	5.7	1.06

Values are given as mean ($n = 2$) \pm standard deviation (absolute value).

Table 3 The known antioxidant active amino acid ($\mu\text{g/mL}$ pooled sample) present in P1-P3 fractions emerging from SCE-separation of <10 kDa fractions from SC (spice-cured fillets), TSp (barrel-salted spice-cured) and TSa (barrel-salted).

Amino Acid	SC			TSp			TSa		
	P1	P2	P3	P1	P2	P3	P1	P2	P3
Histidine	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	13	31	-	-	-	-
Proline	-	-	-	130	-	-	185	-	-
Tyrosine	-	-	-	-	37	-	-	43	-
Total	0	0	0	143	68	0	185	43	0
All amino acids	1219	0	0	3807	128	0	3561	43	30

Values are given as mean ($n = 2$), and all standard deviations are below 1%. Values below detection limit are marked with (-).

Table 4 Peptides identified in the P1-P3 fractions emerging from SCE-separation of <10 kDa fractions from SC (spice-cured fillets), TSp (barrel-salted spice-cured) and TSa (barrel-salted), using MALDI MS and MS/MS analysis. Mass [m/z] of the observed peptides and the amino acids sequence are listed.

Fractions	SC	TSp	TSa
P1	1811.9	1326.5 - SSVD[Q/K] 1425.6 - V[SG/GS]V	n.d.
P2	1046.5 - VY[L/I]HDF 1198.5	n.d.	n.d.
P3	1056.5 - HDF 1070.5 - HDF 1088.5 - HDF	n.d.	n.d.

n.d. no peptides were detected in the representative MS spectrum.

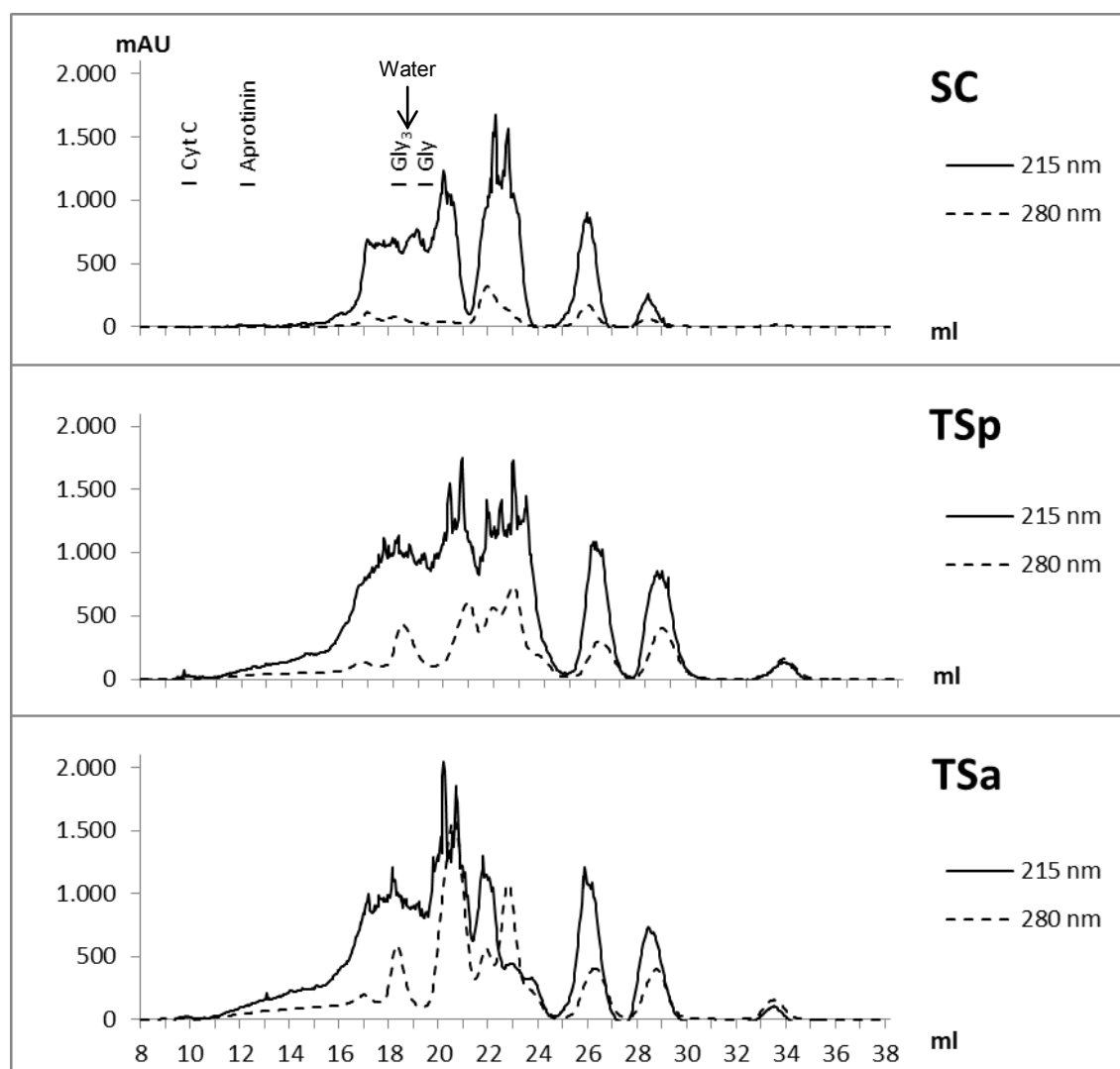


Figure 1

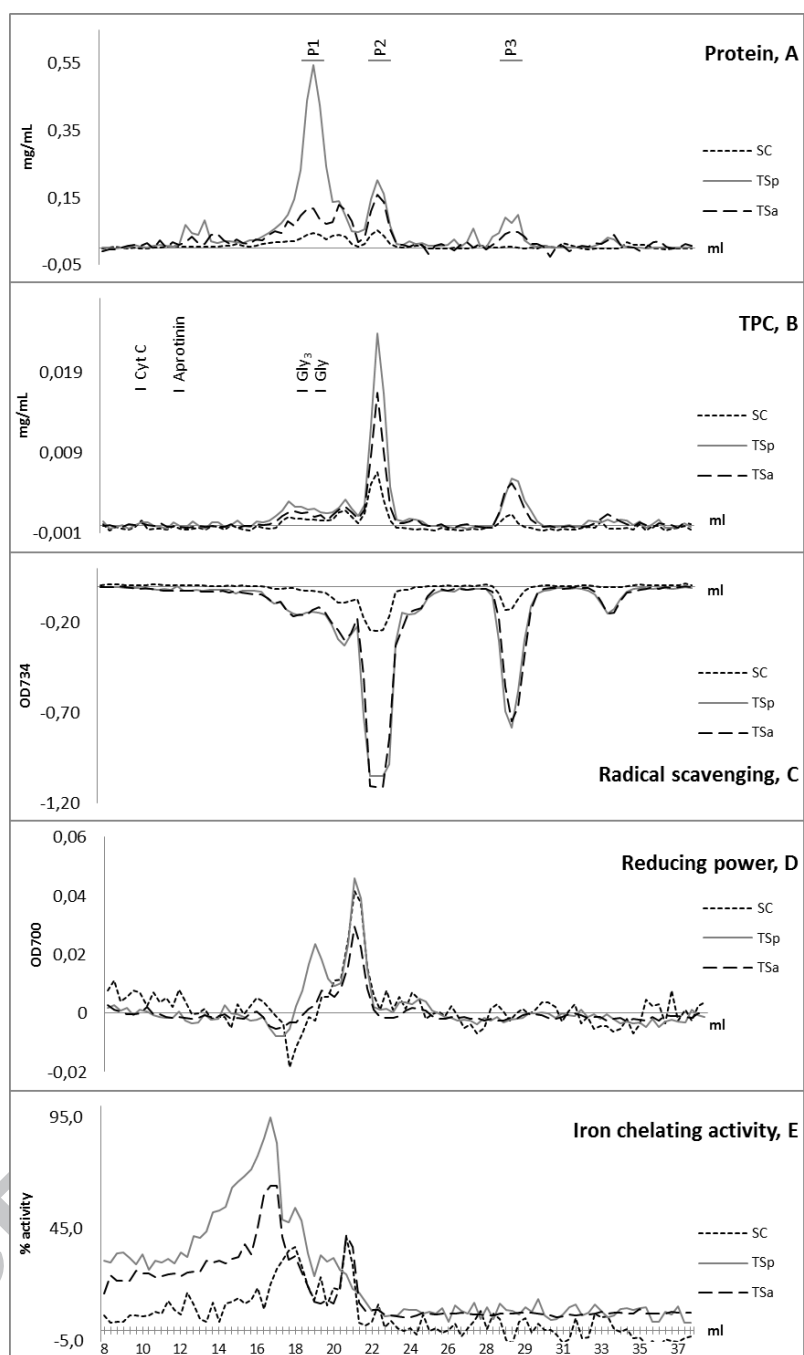


Figure 2

Highlights (3-5, max 85 characters each)

- Herring salt brine contains low molecular weight compounds (LMWC) with antioxidant activities
- LMWC are identified as phenolic compounds and protein/peptides
- Phenolic compounds seem to be mainly responsible for the ABTS-radical scavenging activity
- Peptides seem to be responsible for the iron chelating activity and reducing power
- MS reveals peptides of 1046-1911 Da and presence of known antioxidative amino acids

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Title: Antioxidative low molecular weight compounds in marinated herring (*Clupea harengus*) salt brine

Article Type: Research Article (max 7,500 words)

Keywords: herring brine; LMWC; antioxidant; phenolic compounds; peptides

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Abstract: This study aimed at unravelling the antioxidative capacity of low molecular weight compounds (LMWC) (peptides, amino acids and phenolic acids) present in salt brines from the marinated herring production. Brines were fractionated into <10 kDa fractions using dialysis and further into 94 fractions using size exclusion chromatography. All samples were analysed for protein, total phenolic content (TPC) and antioxidant activities. Protein-enriched samples were pooled (P1, P2 and P3) and analysed for phenolic acids, total amino acids and peptide/protein sequence using advanced mass spectrometry. All salt brines contain LMWC holding ABTS-radical scavenging activity, reducing power and iron chelating activity. Generally, a strong correlation between TPC and ABTS-radical scavenging was found. In contrast, reducing power and iron chelating activity seemed to be caused by peptides. Protein/peptide sequencing revealed 1 kDa peptides with the presence of HDF-motif which could be responsible for some of the antioxidant capacity observed in marinated herring salt brine.

PAPER B

Salted herring brine as a coating or additive for herring (*Clupea harengus*) products – A source of natural antioxidants?

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1 **Salted herring brine as a coating or additive for herring (*Clupea harengus*) products**

2 **– A source of natural antioxidants?**

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9 Running title: Salted herring brine as a coating or additive for herring

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20 **ABSTRACT**

21 The objective of this study was to characterize herring brine and assess their use as natural
22 antioxidant. Herring brine from different marinated products (TSa, TSp and SC) were used without
23 any pre-treatment or with a pH adjustment and were tested as coating agents for frozen herring or as
24 additives in fresh mince herring in order to prevent oxidation.

25 TSa and TSp were the most effective glazing, retarding lipid oxidation as confirmed by both the
26 measurement of peroxide and volatiles oxidation products and were better than water. Brines tested
27 as additive retarded lipid and protein oxidation in a similar trend than herring mince containing salt
28 and/or protein. SC brine was a better as additive against lipid and protein oxidation compared to the
29 other tested brines TSa and TSp. Using protein fractions isolated from herring marinating brines as
30 glazing and/or additive seems feasible for preventing oxidation of both frozen and fresh herring.

31

32 Keywords: Herring, brine, pH-shift, glazing, oxidation, natural antioxidant.

33 **Highlights**

- 34 • All the brines were more effective than water glazing in frozen herring
- 35 • Brines delayed lipid and protein oxidation in fresh herring mince
- 36 • Alkali pH-shift of brines did not produce any advantages compared to unmodified pH
- 37 • TSa and TSp showed the best antioxidant potential as glaze
- 38 • SC was an effective as antioxidant in fresh herring mince

39

40 **1. INTRODUCTION**

41 Barred-salted herring is an important fish product in the Nordic fishery industry whilst in the south
42 of Europe anchovies are more common as salted product. During the salting process a long
43 maturation period takes place, where degradation of proteins occurs due to both digestive and
44 muscle proteases (Nielsen, 1995). During this long ripening period transport of biomolecules such
45 as proteins, lipids and peptides leach out from the fish to the brine (Svensson, Nielsen, & Bro,
46 2004) leading to a brine rich in organic matter. After the ripening period, the maturing brine is
47 removed and discarded and before barred-salted herring's commercialisation the fish is packed with
48 fresh brine containing spices and flavourings. Therefore, during the production very large volumes
49 of marinade with high organic load are discarded. There is therefore a need to demonstrate if these
50 liquid wastes, which contain high-value marine biomolecules such as protein, lipids and peptides,
51 could be re-utilised and valorised. Furthermore, marinating brines, which are a food grade waste,
52 could represent a good source of natural additive such as antioxidants. Herring brines have been
53 characterised and contain proteins and peptides (Gringer, Osman, Nielsen, Undeland, & Baron,
54 2014) which may be able to protect the lipids from oxidative damage under herring ripening
55 (Andersen, Andersen, & Baron, 2007). A recent study characterising brine from different
56 Scandinavian products demonstrated, using *in vitro* tests, that brine possesses radical scavenging
57 activity, iron chelating activity and reducing properties and therefore has the potential to be used as
58 a source of antioxidants (Gringer et al., 2014). Similarly, a study that tested herring press juice, in a
59 model fish system and a simulated gastrointestinal digestion, demonstrated that it possesses good
60 antioxidant capacity, attributed to low molecular weight compounds (Sannaveerappa, Sandberg, &
61 Undeland, 2007a; Sannaveerappa, Carlsson, Sandberg, & Undeland, 2007b). Recently, Taheri,
62 Farvin, Jacobsen, and Baron (2014) isolated proteins fractions from barrel-salted herring brines and
63 reported that they exhibit good antioxidant properties *in vitro* and in simple emulsions system.

64 However, despite their potentially interesting antioxidant properties, no studies have showed how
65 these protein-rich wastes could be directly valorised using minimal and simple procedures such as
66 one-step fractionation and centrifugation.

67 Glazing/coating is a common practice in the seafood industry to preserve frozen fish from oxidation
68 and dehydration and as alternative to this procedure, there is increasing research on edible coating
69 based on proteins (e.g. soy, albumin and whey), fish skin hydrolysates and chitosan, (Sathivel,
70 2005, Sathivel, Liu, Huang, & Prinyawiwatkul, 2007, Gómez-Estaca, Montero, Giménez, &
71 Gómez-Guillén, 2007, Rodríguez-Turienzo et al.,2011). Kakatkar, Sherekar and Venugopal (2004)
72 reported that acidic fish protein dispersion applied as a glaze to frozen fish blocks or fillets had a
73 positive impact on quality reducing oxidation, and dehydration. Another investigation also showed
74 that acidic dispersion of fish protein applied to seer fish improved its microbiological quality
75 compared to water glazing (Phadke, Pagarker, Kumar Reddy, & Kumar Meena, 2012). Herring
76 brines are dispersion of fish protein and no investigation has been performed demonstrating their
77 ability to be used as edible coating to protect frozen fish.

78 One of the processing technologies that have received a great deal of attention in the marine sectors
79 to obtain functional proteins is the pH shift method to prepare protein isolate. In brief, in a first step
80 muscle proteins are homogenised with water and solubilised at low pH ($\text{pH} \leq 3$) or at high pH
81 ($\text{pH} \geq 10.5$). Centrifugation allows separation of insoluble material and in a second step the
82 solubilised proteins are re-precipitated at their isoelectric point and a protein isolate is obtained
83 (Undeland, Kelleher, & Hultin, 2002). This technique has been used to obtain protein isolate from
84 several fish species including fish solid waste (Park, 2012, Chitsomboon, Yongsawadigul, &
85 Wiriayaphan, 2012). Application of the soluble proteins fraction (before precipitation) has been
86 investigated as coating agent and several patents exist demonstrating its use as moisture retention
87 and coating agent preserving fish products quality (Kelleher, 2006, 2011). However, the pH shift

88 has been investigated for sardine stick water as a way to recover solid (García-Sifuentes et al.,
89 2009) but has not been investigated at all on liquid effluents from the fatty fish processing industry
90 such as herring marinades. Therefore, in this study brine protein was solubilised at extreme (2 or 11)
91 pH, freeze-dried and applied to herring. Herring brine was tested either as coating agent for frozen
92 fish or as an additive in fresh herring mince in order to investigate possible routes for valorisation of
93 this liquid waste rich in protein and peptides as a source of natural antioxidants.


94 **2. MATERIALS AND METHODS**

95 **2.1. Brines**

96 2.1.1. Initial brine

97 Brine from traditional barrel-salted herring (*Clupea harengus*) (TSa), traditional barrel-salted spice-
98 cured herring (TSp), and brine from fillet-ripened spice-cured herring (SC) were obtained from the
99 local fish herring processing industry (Lykkeberg A/S, Hørve, Denmark) and selected for
100 valorisation. The pH of the raw brines was measured directly using a Metrohm 780 pH meter
101 (Switzerland). All analyses on the brines were performed in triplicate and in two different sampling
102 days.

103 2.1.2. Acidic and alkaline brine solutions

104 Fifty-millilitre samples of brines were adjusted to pH 2 or pH 11 (acidic or alkaline methods,
105 respectively). All brines were centrifuged (Sorvall RC 5B Plus, Dupont, Norwalk, CT, USA) at
106 11.403 g for 20 minutes at 10 °C. Samples were filtrated through cotton and the collected permeates
107 were freeze dried (Heto DryWinner 8, Thermo Fisher Scientific, broughborough
108 refrigerated at 4 °C until further analysis. The mass balance is expressed as volume yield (final
109 volume of permeate/sample volume after adjusting pH) in percentage (%).

110

111 **2.2. Characterisation of brines**

112 **2.2.1. Protein and salt content**

113 Soluble protein content (mg/mL) of the initial brine and each acidic and basic permeate was assayed
114 using a BCA kit (Thermo Scientific Pierce®, Rockford, USA), with bovine serum albumin (BSA)
115 as standard.

116 The salt content in percentage (w:w) of the initial brine and the acidic and basic permeates was
117 determined using the AOAC standard method (AOAC, 2000).

118 **2.2.2. Protein profile**

119 Brine samples were diluted to 1 mg/mL in 50 mM Tris buffer (pH 7.4) containing 1 mM EDTA, and
120 further mixed 1:1 (v:v) with Laemmli buffer with 10% DDT. Subsequently the mixture was boiled
121 for 3 minutes and centrifuged for 3 minutes at 13.684 g. Samples and standard See-blue (10 µL)
122 were loaded onto a 10% NuPAGE® Bis-Tris Gels (Novex®, 1.0 mm, 12 well, Life Technologies™,
123 Denmark) and run with MOPS (4-morpholinepropanesulfonic acid) running buffer at 200V for
124 approximately 50 minutes). After the run, gels were stained with Coomassie Brilliant blue G-250
125 overnight. Finally, the gels were washed with a destaining solution of 15% ethanol and 5% acetic
126 acid until protein bands became clearly visible in a colourless gel matrix.

127 **2.3. Storage experiments**

128 **2.3.1. Frozen herring coating**

129 Ten kilograms of fresh herring (*Clupea harengus*) were obtained from local fishmongers, filleted
130 with skin on and subsequently vacuum packed and stored at -80 °C until further experiment.

131 Coating was performed at 4 °C and frozen herring fillets were randomly allocated into three
132 batches: initial brine SC, TSa and TSp adjusted to protein concentration of 1 mg/ml. Brief dipping
133 the fish fillet in the fish protein solution for 10 seconds three consecutive times with 15 second
134 intervals resulted in a thin coating layer onto the fillet surface. Controls consisted of non-coated
135 fillets, vacuum packed fillets and fillets coated with water. For each treatment a minimum of 6
136 randomised fillets were used. The fish were placed in aluminium foil and stored for 4 and 10 weeks
137 at -10 °C before they were stored at -80 °C until further analysis.

138 2.3.2. Fresh herring mince

139 Ten kilograms of fresh herring (*Clupea harengus*) were obtained from the local fishmongers,
140 filleted, and skinned and minced using a kitchen blender (Robot Coupe Ra A7S, France). The mince
141 was stored in plastic bags under vacuum at -80 °C until further experiment. Samples of mince (250
142 g) were randomly allocated into the following batches containing 1 g per kg of SC; TSp; TSa
143 powder or their corresponding alkaline TSa pH 11; TSp pH 11; SC pH 11 re-solubilised in water.
144 Added water represented 10% (w:w) of the initial herring mince. Controls samples containing no
145 additive, salt, BSA, or salt + BSA were also used. The content of salt and BSA were 125 and 600
146 mg per Kg of herring mince respectively, which were used because these represent the highest
147 amount of salt and protein in the initial brines. All batches were kept in trays at 5 °C and samples
148 analysed on days 1, 2, 4 and 7 of storage. At each sampling point, the samples were packaged in
149 vacuum plastic bags and stored at -80 °C until further analysis.

150 2.4. Storage stability

151 2.4.1. Lipid oxidation

152 *Total lipid content and peroxide value (PV)*

153 Total lipids extract were obtained from 10 g herring mince or fillet with methanol/chloroform (1:1
154 v/v) according to the method of Bligh and Dyer (1959). PV was measured directly on the lipid
155 extract (LEX) according to the method described by the International IDF Standards (1991) and
156 expressed as milli equivalent (meq) of O₂ per kg oil.

157 *Thiobarbituric reactive substances (TBARS)*

158 Fish and mince samples were analysed for TBARS using the methodology described by Vyncke
159 (1975). Results were expressed as µmol malondialdehyde equivalents (MDA) per kg of muscle.

160 *Volatile secondary oxidation products*

161 The volatile secondary oxidation products were analysed only at day 4 for the herring mince and
162 after 4 and 10 weeks for the glazed herring fillet using dynamic headspace according to Eymard,
163 Baron, and Jacobsen (2009). An aqueous suspension of 10 g of fish powder was purged at 37 °C in
164 a water bath for 30 minutes with a nitrogen flow of 340 mL/min. The volatile compounds in the
165 samples were collected on Tenax GR traps (Chromapack, Bergen op Zoom, The Netherlands). A
166 Perkin–Elmer (Norwalk, CT) ATD-400 automatic thermal desorber system was used for thermally
167 desorbing the collected volatiles from the Tenax traps using helium as a carrier gas (with a flow of
168 1.3 mL of helium/min). Thereafter volatiles were separated and quantified by gas chromatography-
169 mass spectrometry (GC-MS), a DB 1701 column (30x 0.25 mm, 1.0 µm; J&W Scientific, Folsom
170 CA, USA) and the following programmes of temperatures were used: 35 °C for 5 minutes, 35-90 °C
171 at 3 °C min⁻¹, 90-240 °C at 10 °C min⁻¹, and finally hold at 240 °C for 4 minutes. The GC–MS
172 transfer line temperature was kept at 280 °C. The mass-selective detector used ionisation at 70 eV in
173 EI mode and 50 µA emission. The scans were performed in the mass range of 30–350 atomic mass
174 unit with a repetition rate of 2.2 scans/s. The compounds were identified by MS library searches and
175 by comparing retention time and spectra with MS runs of external standards. For quantification

176 purpose, calibration curves were prepared by adding a mixture of ten selected standard in ethanol
177 (concentrations 0.01-0.05 mg/g) directly on Tenax tubes (1 µl) and performing thermal desorption
178 and GC-MS analysis under the conditions described above for the samples.

179 2.4.2. Protein oxidation

180 Protein carbonyls were measured on the herring mince as described by Levine, Williams, Statdman
181 and Shacter (1994). Results are expressed in nmol carbonyls per mg of protein.

182 **2.5. Statistical analysis**

183 The data for pH, mass balance, soluble proteins and salt content were subjected to One-way
184 ANOVA. Data of coating storage and mince herring experiment, with the exception of FAME and
185 volatiles in mince herring, were analysed by multifactor ANOVA of each variable, taking into
186 account treatment and time. Unless otherwise mentioned all analysis were performed in triplicate.
187 Fisher LSD (Least Significant Difference) test was applied for determining group differences at
188 95% of significant level. Stagraphic Centurium XVII was used for carrying out the statistical
189 analysis.

190 **3. RESULTS & DISCUSSION**

191 **3.1. Characterisation of brines**

192 Different brines (SC, TSa and TSp) and their acidic and basic counterparts (pH 2 and pH 11,
193 respectively) were characterised for the biochemical composition (protein, salt concentration and
194 protein profile using SDS-PAGE). The initial pH of the brines was found to be in the range of 5.96
195 to 6.05.

196 The analysis of soluble protein in the initial brines shows the highest amount in TSp (50.8 ± 3.5
197 mg/mL), followed by TSa (41.0 ± 2.68 mg/mL) and SC (37.9 ± 3.15 mg/mL) (**Table 1**). Marinating
198 time is a crucial factor to determine the final amount of total nitrogen, both protein and non-protein.
199 Nitrogen diffuses from herring into the brine during ripening (Szymczak & Kolakowski, 2012). SC
200 had the shortest time of ripening, approximately 9 months, compared to TSa and TSp, which were
201 marinated for more than 16 months and this could explain the difference in protein content in the
202 brines, which has also been reported previously (Gringer et al., 2014).

203 Yield expressed as volume (**Table 1**) ranged from 42 to 56% for unmodified brines, 35 to 49% for
204 acidic- and 60 to 64% for basic-solubilised brine, respectively. Yield decrease can be due to the loss
205 of solubilised proteins into the floating layer and sediments during centrifugation. Protein
206 solubilisation into the floating layer has been reported to be lower for herring compared to other
207 food such as mackerel and chicken (Undeland et al., 2002). The differences were explained by the
208 structure of the acidified/alkalised herring homogenates, which creates a large floating layer and
209 sediment with high water content. In fresh fish, the solubility of muscle proteins can be greater than
210 95% of the total proteins but herring brine contains salt which solubilise some protein (salting in)
211 whilst some others might precipitate (salting out). The theory behind acid or alkaline protein
212 isolates is based on the formation of net, respectively positive or negative charge on the proteins,
213 and consequently electrostatic forces are created, which drive the molecules apart from each other,
214 enhancing solubilisation. The largest protein yield was obtained for the basic pH treatment,
215 followed by neutral pH and acid pH. Compared to the untreated brines the acidic treatment resulted
216 in 70% less protein whilst the basic treatment led to almost 100% of protein recovery. It can be
217 concluded that solubilisation of protein from herring brine at pH 2 is not very efficient. A possible
218 cause for this behaviour could be due to the low pH used which is lower than those found in
219 literature and in agreement with reports from Marmon and Undeland (2013), who reported that a pH

below 2.5 decreased herring muscle protein solubility. Contrary to our results, protein recovery yield by acid solubilisation is usually higher than by alkaline solubilisation (Nolsøe & Undeland, 2009). Despite the low pH selected for our solubilisation, another explanation could be based on the high amount of salt contained in the samples, and a possible interaction between salt and pH, as reported by Kim and Park (2008) with Alaska pollock protein isolates, where salt induced salting out of unfolded protein. The salt content for all samples ranged between 12 to 15% (data not shown). Alkaline solution had significantly ($P<0.05$) lower salt concentration (12 to 13%) than acid solutions (15 to 13%), and both significantly lower than the unmodified brines (16-14%). NaCl is known to increase muscle protein solubility, even if the solubility of thin and thick filament protein is largely different, salt affects the pH shift solubilisation of protein. Interaction between salt and protein and the impact of salt on protein unfolding and solubility in muscle food is still a major debate (Puolanne & Halonen, 2010). Analysis of the protein profiles through SDS-PAGE (**Figure 1**) was performed for initial and alkaline treated brines, as the acid treated samples were discarded from further studies due to their low protein yield. For all unmodified and alkaline solutions studied, myosin did not appeared in any sample as no band was visible at around 200 kDa, being probably extensively degraded during the processes. Despite the amount of scientific literature describing the fish marinating process, there is a lack of information on the qualitative and quantitative protein that is transferred to the brine during the marinating process (Szymczak & Kolakowski, 2012). Clear differences in the pattern of protein molecular weights profiles are observed between the SC permeates and TSp and TSa. In general, SC showed higher molecular weight protein than the other two types of brines (TSp and TSa) which can be attributed to the high amount of proteolytic enzyme in these samples compared to SC (Gringer et al., 2014). SC showed an intense fragment around 45 kDa and another around 49 kDa, but did not show the intense band revealed by TSp and TSa profiles at approximately 37 kDa. This different protein pattern was also

244 in agreement with previous results (Gringer et al., 2014). For TSa and TSp lighter bands were
245 observed in the high molecular weight region above 100 kDa. However, for all sample, the alkaline
246 treatment seemed to decrease the amount of polypeptides identified in the region between 45 and 36
247 kDa. Similar results were reported by Undeland et al. (2002) and Vareltzis, Adamopoulos, and
248 Hultin (2011).

249 **3.2. Frozen herring coating**

250 Rancidity during storage for frozen herring fillets coated with brine (SC, TSa, TSp) was measured
251 by primary and secondary oxidation products: peroxide values (PV) and volatiles, respectively.
252 Herring average oil content used in this experiment was 20% (wet weight). PV showed early lipid
253 oxidation (**Figure 2**) and after 4 weeks of storage a protective effect of brine glazing was observed
254 when comparing PV to non-coated samples. Brine seemed more potent to act as an antioxidant
255 compared to water coating. These differences between PV of coated and un-coated samples were
256 more evident after 10 weeks of storage. PV of TSp and TSa brine-glazed samples were significantly
257 ($P<0.05$) lower than those of SC samples, and protein and peptide content (higher in TSp and TSa
258 brines than in SC brine) may have contributed to this effect. A recent report (Gringer et al., 2014)
259 also reported that TSa and TSp had higher iron chelating and radical scavenging activities than SC,
260 which could contribute to their ability to be better coating agent than SC and water. Other authors
261 (Kakatkar et al., 2004; Rodríguez-Turienzo et al., 2011) also reported that the protein glazing
262 limited the lipid oxidation in fishery products during frozen storage and were a better protection
263 than water glazing. However, more investigations are needed to reveal the exact mechanisms.
264 Coatings of salmon with whey protein isolate and acetylated monoglyceride also reduced lipid
265 oxidation during frozen storage (Stuchell & Krochta, 1995). It was further suggested that protein
266 coating reduced the diffusion of oxygen and the availability of oxygen to the fish surface. It is
267 possible that in herring brine, a combination of salt and protein change the structure of the frozen

268 water on the surface of the fish allowing less oxygen to penetrate the muscle tissues thereby
269 preventing oxidation. Protein solutions have been the subject of patents (Kelleher 2006, 2011) in the
270 process of protein isolate and have been applied to acidic solutions of muscle protein or dry
271 powders into foods to retain moisture during cooking, prevent fat absorption during frying and
272 prevent dehydration among other effects. Herring brines are acidic with pH value around 6 and
273 contain large amount of protein and peptide and therefore can also be considered for such
274 applications and more investigations are needed to demonstrate their potential. The concentration of
275 volatile secondary products such as 1-penten-3-ol, hexanal, heptanal and 2,4-heptadienal after 4 and
276 10 weeks of storage are shown in **Table 2** and these results are in agreement with those for primary
277 oxidation products (PV). After 4 weeks, although not significantly ($P < 0.05$), the level of 2,4-
278 heptadienal was lower in brine glazed fillets compared to water glazed, whilst no difference was
279 observed for the other analysed volatiles. However after 10 weeks, TSa and TSp seemed to protect
280 better against oxidation than water glazing. In general, these results suggest that the use of herring
281 brines can represent an alternative to the traditional water glazing allowing the product to be stable
282 for a longer period of time. The mixture of compounds present in the brine such as organic acids,
283 natural antioxidants from the fish or inherent to the spices added (SC and TSp) during processing of
284 marinated herrings, protein and peptide with antioxidant activity, makes it difficult to access the
285 exact nature of the active compounds. Elias, Kellerby, and Decker (2008) reviewed the antioxidant
286 activity of protein and peptides and indicated that hydrolysis with the formation of peptides lead to
287 higher antioxidant properties, which could explain differences between TSa and TSp compared to
288 SC. Nevertheless, more research is needed including purification, structure identification and
289 investigation of the mechanisms responsible for the antioxidative activity in herring brines.

290 **3.3. Fresh herring mince additive**

291 The impact of addition of brine or their basic counterpart as additive was tested during storage of
292 herring mince and the results for lipid (PV and TBARS) and protein oxidation (protein carbonyls)
293 are presented in **Table 3**. PV indicated higher levels of lipid oxidation in all alkaline-solubilised
294 brine compared to initial brine, a trend which was also confirmed with other lipid oxidation
295 parameter such as TBARS. Among samples, SC showed lower PV compared to TSp and TSa. The
296 alkalinisation process did not lead to any additional antioxidant activity; on the contrary, it led to
297 more unstable herring mince when evaluating TBARS and PV. This might be explained by the
298 results from Vareltzis et al. (2011) demonstrating that strong pro-oxidants, such as heme protein
299 which are known to be present in the brine, are less bound to membrane and thus more pro-
300 oxidative. Compared to the control without any additive, SC, TSa and TSp had lower PV and
301 TBARS from day 1, indicating that as an additive, brine is able to prevent lipid oxidation. However,
302 there were no significant differences between samples and no significant difference when compared
303 with sample with salt and/or protein. Significant difference was observed after 4 days of storage,
304 however in PV for TSp only when compared to no additive. In contrast, all brines seemed to present
305 some antioxidant potential when examining TBARS. After 4 days, the oxidative stability order was
306 SC > TSp > TSa. However, the brine samples were comparable to salt and/or protein added
307 samples. Both protein and salt played a protective role toward oxidation, but a non-synergistic
308 effect was found when salt and protein were added together at the concentration used. Several
309 research papers have showed the protective effect of protein toward lipid oxidation and it is well-
310 known that proteins and peptides can have antioxidant activities (Elias et al., 2008). At day 7, SC,
311 TSa and TSp appeared to be preventing oxidation, and among their basic counterpart only SC
312 seemed to show this effect. Peptides obtained from alkaline isolate hydrolysates from tilapia muscle
313 proteins have been shown to possess some antioxidant activity with increasing degree of hydrolysis,
314 but extensive hydrolysis is also known to reduce the antioxidative potential (Raghavan &

315 Kristinsson, 2008). This could explain the loss of antioxidative activity in TSa and TSp compared to
316 SC. Indeed, the traditional barrel-salted process method (TSp and TSa) includes a step where the
317 headed fish is stored for 6 months in brine, which can result in the transport of hemoglobin and
318 other pro-oxidative compounds from the fish into the brine, but the process is also known to induce
319 hydrolysis of the muscle protein due to the presence of proteases. The basic treatment of the brine
320 might have further induced protein and peptide hydrolysis in TSa and TSp up to an extent that
321 resulted in a loss of activity.

322 The formation of carbonyl compounds from amino acid side chains is probably the most
323 outstanding result of metal-ion catalysed oxidation of myofibrillar protein (Lund, Heinomen, Baron
324 & Estévez, 2011). Initial carbonyl value (unprocessed herring minced) was 0.56 mmol/g of protein.
325 As expected, the formation of protein carbonyls increased over storage time. Carbonyl content was
326 significantly greater in herring with alkalised brines. These results differ from Marmon and
327 Undeland (2013); these authors did not find protein oxidation as measured by carbonyl content as a
328 consequence of the alkaline pH-shift processing. The numerical value of carbonyls was
329 significantly lower in minced herring containing SC brines compared to TSp and TSa. Protein
330 oxidation are in agreement with lipid oxidation results. This may be due to the different process to
331 obtain the SC brine where fillets are marinated in brine compared to the TSa and TSp where the
332 headed fish is processed. According to our results, samples without any additives showed the
333 highest carbonyl value, while including salt prevented protein oxidation. This last result is in
334 contrast with the findings by Kanner, Harel and Jaffe (1991), which showed a pro-oxidative effect
335 of salt. In this regard, Andersen et al. (2007) stated that salt can be either antioxidative or pro-
336 oxidative in muscle food. The brines in ranking order of antioxidant capacity when considering both
337 protein and lipid oxidation are SC > SC pH 11 > TSp > TSa. These results differ from the glazing
338 experiment where TSa and TSp were better antioxidants than SC, demonstrating that the application

is an important part of the antioxidant potential and that antioxidant activity should always be tested in a matrix based set-up/experiment, as also suggested by others Farvin, Grejsen and Jacobsen (2012). Evaluation of volatile secondary oxidation products with identification and quantification in the herring mince was performed on day 4 and the results are presented in **Table 3**. The alcohol 1-penten-3-ol is one the major oxidation products in herring mince among volatiles analysed and our results are in accordance with Sampels, Åsli, Vogt, and Mørkøre (2010). Hexanal and 1-penten-3-ol have been identified as good markers for early lipid oxidation and correlated with rancid off-flavour. Hexanal is representing the omega-6 whilst 1-penten-3-ol is representing the omega-3 fatty acid degradation products. The volatiles analysed at day 4 all showed a similar tendency. SC had the lowest concentration of volatiles while the highest levels were found in TSp, both unmodified and basic pH-solubilised. Among controls, there were only significant differences between samples without additive and the rest of the samples (i.e. salt, BSA and salt+BSA) in the case of hexanal and 1-penten-3-ol where protein (BSA) and salt could have played a protective role preventing lipid oxidation. The results observed were in agreement with the data in **Table 2** indicating a ranking order from lowest to highest presence of volatiles related to lipid oxidation as following: SC > SC pH 11 > TSp > TSa.

4. CONCLUSIONS

In summary, this investigation revealed the possibility of using herring brines by-products as natural antioxidant coating agents or additives. The use of brines as a glaze for frozen herring could represent an alternative to water glazing especially for TSa and TSp. Hence, this by-product can be used as natural protection for frozen fish fillets in fish prone to oxidation such as herring. Tested as antioxidant ingredient in herring minced, the brines in all cases showed a very good antioxidant activity when compared to the control. However, the salt and protein controls also showed a good antioxidant effect. Nevertheless, in herring mince SC brine was very effective in retarding lipid and

363 protein oxidation as it resulted in low level of PV, TBARS, volatiles and carbonyls. Alkaline pH-
364 solutions did not show any further benefit compared to unmodified brines. The direct use of protein
365 solutions isolated from herring marinating brines seems feasible for preventing the oxidation of
366 fresh herring mince, although it seems that salt and protein present in the brine play a key role in the
367 observed antioxidant effect. Nevertheless the exact nature of the antioxidants in the brine still needs
368 to be revealed. Furthermore the brines showed different behaviour depending on the application,
369 with SC being better suited for mince and TSa and TSp better suited for coating of frozen fish.
370 Thus, the results of the present study suggest the use of brines can be successfully employed as
371 coating/glazing and natural ingredient to prevent oxidation in frozen herring fillets and chilled
372 herring mince respectively demonstrating that this waste can be turned into a resource.

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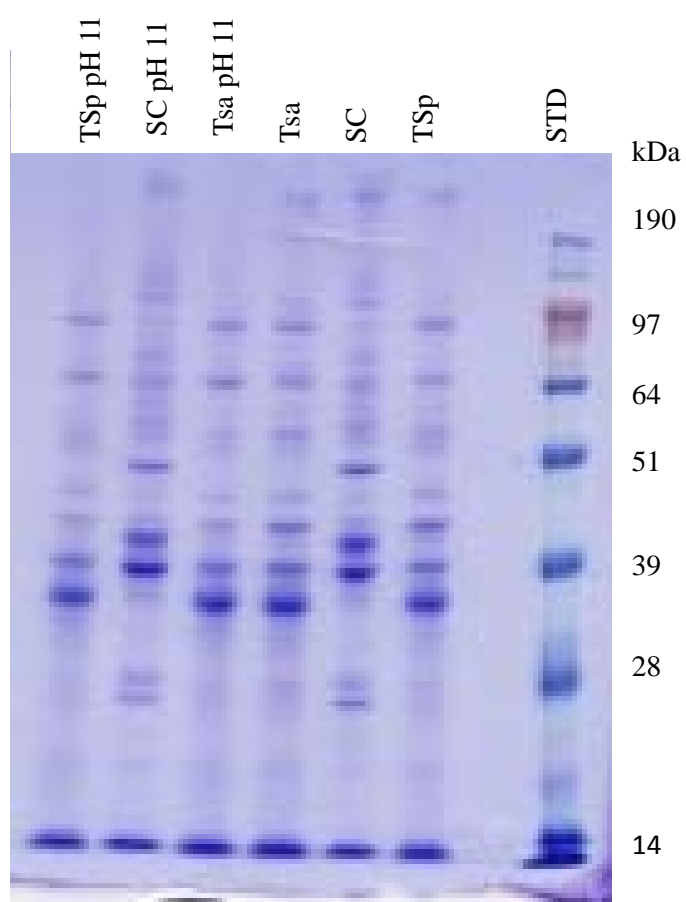


Figure 1: SDS-PAGE of initial brines TSa, TSp and SC and their alkaline (pH 11) solutions. STD: prestain standard seablue with indicated molecular weight kDa.

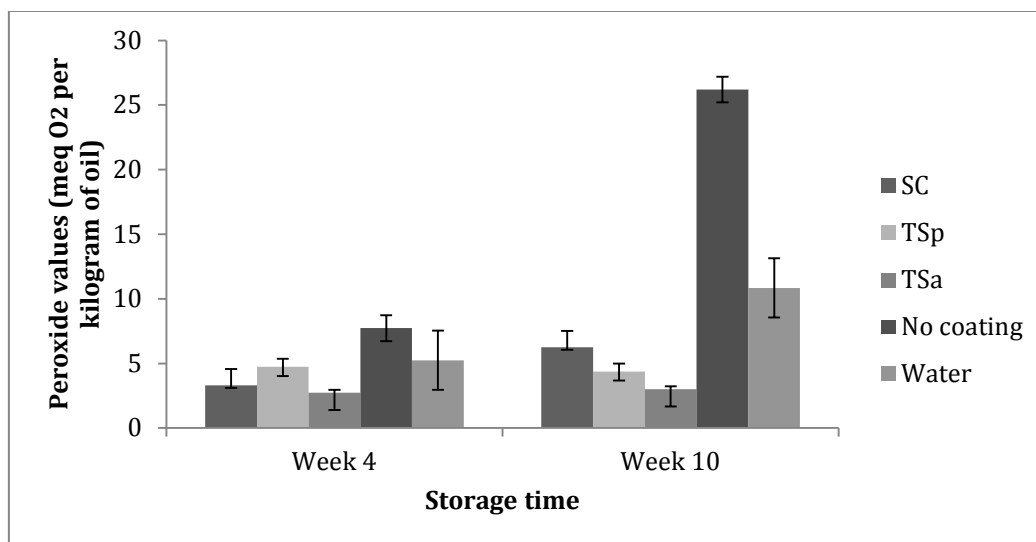


Figure 2: Bars represent the peroxide value (PV) (meq of O₂ per kilogram of oil) during storage of brine coated frozen fillets at -10°C for 4 and 10 weeks from SC, TSa, TSp, Water coating and no coating. Control sample, fillets vacuum packed at -80 °C upon arrival to the laboratory, PV: 0.82 meq per kg oil.

Table 1: Yield expressed in % of initial volume before centrifugation and soluble protein expressed in (mg/ml) of the recovered brine SC, TSa, TSp and their acidic (pH 2) and alkaline (pH 11) solutions.

	Yield (%, v:v)	Soluble protein (mg/ml)
SC	56.67(±4.16)cdf	37.95(±3.15)d
TSa	42.00(±3.46)ab	41.08(±2.68)d
TSp	52.00(±7.2)bcd	50.83(±3.56)f
SC pH2	49.08(±7.83)ab	2.73(±0.57)a
TSa pH2	47.72(±1.49)bc	3.95(±0.77)a
TSp pH2	35.44(±2.13)a	22.91(±4.56)b
SC pH 11	64.04(±9.85)f	30.17(±1.98)c
TSa pH 11	60.20(±0.71)df	34.13(±3.32)cd
TSp pH 11	63.51(±5.29)f	37.77(±3.28)cd

Values (mean ± standard deviation. n=3) followed by the same lowercase letter in same column are not significantly different (P<0.05).

Table 2: Development of volatiles (ng per g minced) during storage of brine coated frozen fillets at -10°C for 4 and 10 weeks.

	Hexanal		1-penten-3-ol		2,4 heptadienal		Heptanal	
	4 Weeks	10 Weeks	4 Weeks	10 Weeks	4 Weeks	10 Weeks	4 Weeks	10 Weeks
SC	^A 119.0 ^{ab} (±8.6)	^A 181.6 ^{ab} (±59.9)	^A 72.9 ^{ab} (±20.5)	^A 92.7 ^a (±29.5)	^A 99.6 ^a (±21.4)	^B 240.8 ^b (±58.1)	^A 2.2 ^{ab} (±0.6)	^A 7.6 ^{ab} (±5.8)
TSp	^A 104.2 ^{ab} (±29.7)	^A 132.1 ^a (±49.8)	^A 75.60 ^{ab} (±6.1)	^A 69.6 ^a (±12.7)	^A 109.8 ^a (±40.5)	^A 161.8 ^{ab} (±18.0)	^A 1.4 ^a (±0.5)	^A 3.5 ^a (±1.3)
TSa	^A 76.5 ^a (±23.7)	^A 165.9 ^{ab} (±69.6)	^A 50.03 ^a (±17.0)	^A 70.3 ^a (±15.7)	^A 81.5 ^a (±9.5)	^A 127.2 ^a (±36.1)	^A 1.2 ^a (±0.5)	^A 3.2 ^a (±1.6)
No Coating	^A 156.8 ^b (±49.6)	^A 278.2 ^b (±102.6)	^A 83.3 ^b (±8.2)	^A 177.2 ^b (±82.3)	^A 232.7 ^b (±23.9)	^A 320.0 ^c (±63.7)	^A 3.6 ^b (±1.0)	^A 15.6 ^b (±10.0)
Water	^A 73.1 ^a (±41.7)	^A 149.8 ^{ab} (±64.1)	^A 56.3 ^a (±18.3)	^B 112.7 ^{ab} (±8.8)	^A 112.3 ^a (±59.3)	^A 124.3 ^a (±28.7)	^A 1.8 ^a (±2.3)	^A 4.8 ^a (±3.7)
Vacuum Packed*	22.8 (±1.6)		26.6 (±5.5)		32.5 (±6.0)		0.3 (±0.1)	

*Control sample, fillets vacuum packed at -80 °C upon arrival to the laboratory.

Values (mean ± standard deviation. n=3) followed by the same uppercase letter in same row are not significantly different (P<0.05).

Values (mean ± standard deviation. n=3) followed by the same lowercase letter in same column are not significantly different (P<0.05).

Table 3: Carbonyls content (nmol per mg of protein), Peroxide value (PV) (meq per Kg of oil) and Thiobarbituric Acid Reactive Substances (TBARS) (μmol malondialdehyde (MDA) per Kg of muscle) for herring minced stored at 5°C and added 1000 mg/kg of SC, TSa, TSp brine or their alkaline pH 11 counterparts or 125 mg per Kg salt (NaCl) and/or 600 mg per Kg bovine serum albumin (BSA).

	Day 1			Day 4			Day 7		
	Carbonyls	PV	TBARS	Carbonyls	PV	TBARS	Carbonyls	PV	TBARS
SC	$1.27 \pm 0.05^{\text{ab}}$ $1.27 \pm 0.05^{\text{ab}}$	$4.05 (\pm 1.09)^{\text{abc}}$	$66.44 \pm 1.7^{\text{cdf}}$	NM	$7.33 (\pm 0.44)^{\text{a}}$	$71.88 \pm 1.62^{\text{ab}}$	$3.43 \pm 0.53^{\text{a}}$	NM	$73.25 \pm 9.51^{\text{ab}}$
TSp	$3.53 \pm 0.09^{\text{d}}$	$5.99 (\pm 0.25)^{\text{df}}$	$44.35 \pm 2.77^{\text{a}}$	NM	$8.53 (\pm 0.52)^{\text{ab}}$	$79.41 \pm 1.87^{\text{bc}}$	$6.49 \pm 0.30^{\text{abc}}$	NM	$92.13 \pm 6.58^{\text{bcd}}$
TSa	$1.27 \pm 0.13^{\text{ab}}$	$2.93 (\pm 1.55)^{\text{a}}$	$41.68 \pm 3.69^{\text{a}}$	NM	$9.83 (\pm 2.43)^{\text{abc}}$	$81.48 \pm 1.87^{\text{bc}}$	$5.52 \pm 0.14^{\text{ab}}$	NM	$84.05 \pm 7.71^{\text{abc}}$
TSa pH 11	$5.29 \pm 0.54^{\text{f}}$	$4.65 (\pm 0.40)^{\text{bcd}}$	$49.43 \pm 4.26^{\text{ab}}$	NM	$10.17 (\pm 0.23)^{\text{abc}}$	$94.17 \pm 1.65^{\text{a}}$	$7.97 \pm 1.41^{\text{bc}}$	NM	$121.61 \pm 1.69^{\text{f}}$
TSp pH 11	$2.65 \pm 0.53^{\text{f}}$	$5.12 (\pm 0.16)^{\text{bcd}}$	$65.38 \pm 3.80^{\text{cdf}}$	NM	$18.8 (\pm 2.22)^{\text{d}}$	$102.44 \pm 4.67^{\text{f}}$	$8.27 \pm 1.09^{\text{c}}$	NM	$105.06 \pm 1.62^{\text{df}}$
SC pH 11	$2.24 \pm 0.19^{\text{bc}}$	$4.52 (\pm 0.61)^{\text{abcd}}$	$75.10 \pm 1.86^{\text{f}}$	NM	$13.51 (\pm 0.23)^{\text{c}}$	$63.15 \pm 1.66^{\text{a}}$	$7.22 \pm 0.17^{\text{abc}}$	NM	$68.39 \pm 6.10^{\text{a}}$
Salt	$1.96 \pm 0.22^{\text{ab}}$	$2.99 (\pm 0.11)^{\text{a}}$	$70.99 \pm 2.40^{\text{df}}$	NM	$8.43 (\pm 1.00)^{\text{ab}}$	$87.51 \pm 1.96^{\text{cd}}$	$4.32 \pm 0.03^{\text{abc}}$	NM	$91.89 \pm 1.82^{\text{bcd}}$
BSA	$3.28 \pm 0.93^{\text{cd}}$	$5.43 (\pm 0.59)^{\text{cdf}}$	$60.99 \pm 0.57^{\text{cd}}$	NM	$8.60 (\pm 0.78)^{\text{ab}}$	$83.65 \pm 2.38^{\text{c}}$	$8.27 \pm 0.49^{\text{bc}}$	NM	$82.99 \pm 1.55^{\text{abc}}$
Salt+BSA	$1.25 \pm 0.05^{\text{a}}$	$3.70 (\pm 0.30)^{\text{ab}}$	$65.14 \pm 5.44^{\text{cdf}}$	NM	$12.14 (\pm 3.36)^{\text{bc}}$	$98.62 \pm 1.39^{\text{f}}$	$5.78 \pm 0.15^{\text{abc}}$	NM	$94.80 \pm 9.15^{\text{cd}}$
No Additive	$1.66 \pm 0.06^{\text{ab}}$	$6.34 (\pm 0.58)^{\text{f}}$	$57.28 \pm 5.30^{\text{bc}}$	NM	$11.84 (\pm 1.62)^{\text{bc}}$	$91.57 \pm 5.33^{\text{cd}}$	$13.77 \pm 0.09^{\text{d}}$	NM	$117.42 \pm 1.34^{\text{f}}$

NM: not measured. Values (mean \pm standard deviation, n=3) followed by the same uppercase letter in same row are not significantly different (P<0.05). Values (mean \pm standard deviation, n=3) followed by the same lowercase letter in same column are not significantly different (P<0.05).

Table 4: Development of volatiles (ng per g minced) for herring mince stored at 5°C 4 days after addition of 1 g/kg of SC, TSp and TSa or their alkaline (pH 11) lyophilized brines. Salt (NaCl) 125 mg per Kg and/or bovine serum albumin (BSA) 600 mg per Kg

	Hexanal	1-penten-3-ol	2.4 heptadienal	Heptanal
SC	15.97(±1.32) ^{bc}	154.75(±14.91) ^{ab}	3.71(±0.07) ^a	5.02(±0.32) ^{abc}
TSp	23.39(±1.64) ^d	218.92(±13.20) ^{cdf}	6.37(±0.04) ^{cd}	7.96(±1.18) ^d
TSa	14.58(±1.49) ^{abc}	205.61(±9.84) ^{bcd}	4.78(±0.41) ^{ab}	6.04(±0.10) ^{bcd}
TSa pH 11	16.76(±2.70) ^{bc}	174.55(±31.41) ^{abc}	5.32(±0.82) ^{bc}	4.40(±0.27) ^{ab}
TSp pH 11	24.98(±6.50) ^d	265.81(±77.39) ^f	7.51(±1.07) ^d	7.15(±0.26) ^{cd}
SC pH 11	14.33(±0.80) ^{bc}	179.84(±20.39) ^{bc}	4.79(±0.44) ^{ab}	7.06(±0.25) ^{cd}
Salt	14.02(±0.40) ^{abc}	149.50(±8.16) ^{ab}	4.12(±0.26) ^a	3.38(±0.14) ^a
BSA	12.10(±1.51) ^{ab}	159.50(±4.74) ^{ab}	3.88(±0.25) ^a	3.51(±0.40) ^{ab}
Salt+BSA	9.70(±1.14) ^a	120.35(±11.28) ^a	4.36(±0.74) ^{ab}	4.71(±0.46) ^{ab}
No additive	16.72(±0.60) ^c	237.40(±19.83) ^{df}	4.09(±0.75) ^a	3.02(±0.11) ^a

Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same column are not significantly different (p<0.05).

6. General discussion

The use of herring by-products as new resources is still a focus area for several research groups (e.g. Taheri et al., 2014; Šližytė et al., 2014; Sørensen et al., 2015). Nevertheless, the work done in this PhD has covered an area which has not been the focus elsewhere. While Taheri et al. (2014) was focusing only on the TSa brine and Sørensen et al. (2015) only on the VC brine, this PhD were considering both TSa and VC plus the two spice versions; TSp and SC, as well as the two desalting brines; D-TSa and D-TSp. The experimental work conducted in this PhD is disseminated in three main papers (I-III) and two supporting papers (A and B) presented in the previous chapter, and the joint discussion of the work is given here.

Paper A was focusing on the process waters generated during the first step in the production of marinated herring; RSW, SW, PW and SB, see **Figure 2.a**. To the best of our knowledge, these process waters have not previously been investigated, and the results obtained led to the conclusion that both the SW and the SB generated could be an interesting novel source of n-3 LC-PUFA as well as a good source of protein. We showed that SB were the process water with the highest content of protein (<12 mg/mL) and fatty acids (<2.5 mg/mL), whereas SW was the richest source of n-3 LC-PUFA; 44.5% of the total fatty acids were EPA and DHA. In total, we estimated that ~9.2 kg protein and ~4.1 kg fatty acids are lost per ton of processed herring, equal to ~20 and ~15% of the total herring protein and fatty acids, respectively. Consequently, our findings here suggest that there are biomolecules of interest in these wastewaters, which could potentially result in an additional revenue for the herring producer.

Furthermore, the two selected separation techniques, EF and ceramic UF, were also tested on these processing waters. We managed to recover up to 80% of the protein and fatty acids, and 70% COD retention were obtained. As discussed in **Section 3.2.4** this level of recovery and retention is in the same range, or even better, than reported by others (e.g. Afonso et al., 2004; Cassini et al., 2010), and we therefore considered the separation as satisfactory although the membranes were clogging and the flux was very low. Consequently, before this treatment is ready as an industrial applicable technique, and can be implemented in the local herring production, this separation technique should be further optimised.

In Paper I, we focused on all types of ripening brines, generated from barrel to glass jar, from four types of end-product; VC, SC, TSa and TSp as well as the two desalting brines from the traditional barrel-salted versions; D-TSa and D-TSp. Although VC is the brine produced in highest quantities (Lykkeberg, 2014), noteworthy amounts of TSa, TSp and SC are produced and these brines turned out to contain significantly more protein and antioxidant as well as enzymatic activity than VC. In total, we estimated that up to 110 kg protein and 40 kg fatty

acids are lost per ton of processed herring during the final ripening step, and as a result the potential for utilisation of these brines is even higher than for the processing waters investigated in Paper A. Together, the results from Paper A and I reveal that per ton herring landed and processed into marinated herring products (from boat to jar), up to 119 kg protein and 44 kg fatty acids are today discarded with the wastewater. With the numbers presented in **Chapter 2** of landed herring in Denmark in 2012, this correspond to an annually loss of ~14.9 ton protein and ~5.5 ton fatty acids, assuming that all landed herring are processed into marinated products (Statistics Denmark, 2013).

As reviewed in **Chapter 2** the salt content, long ripening period and the presence of endogenous enzymes all contribute to the protein degradation in the traditional barrel-salted herring resulting in leaching of protein to the brine. However, the brine from the SC almost resembled TSp in protein level and antioxidant activity as well as protease activity. In contrast to TSa, TSp and SC, the brine from the VC had relatively low protein content but the highest fatty acids content, 20.1 mg/mL. However, VC had almost the same level of reducing power and radical scavenging activity as the SC brine, which might be due to the relatively high level of the amino acids His, Phe, Pro and Tyr in VC, which, as discussed in **Section 4.2**, are known antioxidative amino acids. These antioxidative amino acids could be recovered and utilised, however, this was not tested in this thesis and might thus be the subject of a future study.

Besides discovering the potential in the use of the four ripening brines, Paper I revealed that the two desalting brines, D-TSa and D-TSp, were still highly loaded with antioxidant and enzymatic activity, although the protein content was reduced 3 and 10 times compared to TSa and TSp, respectively. This indicates that the antioxidant activity is not solely related to the protein load but that small molecules leaching out from the herring may contribute to this activity. Regarding iron chelating activity and protease activity, the desalting brines were just as active as their corresponding ripening brines. However, when considering both reducing power and radical scavenging activity, D-TSa were almost as active as TSa, whereas D-TSp were not as active as TSp. Additionally, the total fatty acids content in D-TSp and D-TSa was identical with the values found in TSp and TSa, respectively. This highlights the potential for recovering bioactive compounds in these desalting brines, which to our knowledge has not been studied before and thus deserves more attention.

In Paper II, the electro-flocculation (EF) and ceramic ultrafiltration (UF) technologies were investigated for separation of the two spice-cured herring brines; TSp and SC. As discussed in **Section 3.2.1**, the EF method is a fast and easy method to generate flocs and thus separate the HMW compounds, which flotates with the generated flocs, from the LMW compounds, which stays in solution in the treated water. However, we show that this method is not applicable as a recovery method, as high aluminium concentrations (<1.7 g/L) were

observed in the treated water. Consequently, only UF were considered further using a 50 µm polypropylene filter (PF) as a pre-filtration step to remove larger particles. The issue with accumulated aluminium in the treated water are, to our knowledge, not addressed in the studies using EF for recovery. For instance, Xu et al. (2002) used aluminium-based EF to recover protein and fats from wastewater generated during egg processing, but the level of aluminium in the treated water (recovered fraction) were not commented on. The level of aluminium detected in our study is too high considering that the exposure of aluminium has been described in connection with the development of Alzheimer's disease (Bondy, 2014), and is therefore considered a health hazard. With this in mind, it is believed that when using aluminium-based electro-flocculation for recovery of biomolecules intended for food, feed or nutraceuticals, the level of aluminium in the recovered fraction should be considered. As a consequence, our work focused only on the combined PF and UF treatment. Another option could have been to try other types of electrodes, for instance iron. However, as discussed in **Section 4.2**, transition metal ions like Fe^{2+} can trigger oxidation by initiating the generation of ROS, and thus using an iron electrode in EF should be investigated together with oxidation studies to evaluate if this treatment could be applicable as a recovery treatment. This switch in electrode was, however, not possible within the timeframe of the project, but might be worth testing in the future as the EF was otherwise a fast and easy technology as pre-treatment to UF.

The consecutive PF and UF treatment showed the potential for membrane separation in the recovery of bioactive compounds from marinated herring brines; in total, retentions of up to 42% COD, more than 95% TSS, more than 85% iron, up to 44% nitrogen, more than 100% phosphorus, 100% fatty acids and n-3 LC-PUFA, and more than 80% protein were obtained. Furthermore, the membranes retained none of the phenolics in TSp whereas 40% of the phenolic compounds were retained from SC. Even though acceptable organic load reduction was obtained, and the fatty acids were completely separated from the UF-permeate, optimisation of the treatment is still needed to obtain an acceptable flux and a shorter process time and consequently a better recovery. It should be stressed though, that this work aimed to test the concept of recovering the biomolecules from herring industry process waters rather than optimising the recovery processes. Nevertheless, continuing testing separation techniques for herring brines, with recovery as the purpose, some of the physical and thermal separation techniques presented in **Section 3.2** and **Figure 3.b**, could be applied. For instance, screening could be applied as pre-treatment to the subsequent PF and UF. In the experimental set-up we applied a normal house-holding sieve was used. A more defined set of screens with decreasing sizes may improve the pre-treatment in such way, that the fouling of the following membrane units would be less pronounced. Eliminating the fouling would allow for an acceptable flux making this method appropriate as an online screening and separation unit applicable for the recovery of bioactive compounds from the herring brine.

Another option could be to apply centrifugation, e.g. a tricanter centrifuge. Recently, Šližytė et al. (2014) tested two different processes for extracting high quality food-grade protein and oils from herring by-products; a thermal extraction to separate oil and protein (in stick water) and an enzymatic hydrolysis to produce FPH and separated oil. In both set-ups they used a tricanter to separate the stick water/FPH, the sludge and the oily phase. Although this technology was tested on solid by-product, and not wastewater, the tricanter was efficient in separating the three phases, and might be just as efficient as a separation technique for the herring brines.

No matter which pre-treatment is applied; screening, tricanter or something else, a membrane separation process may be applied afterwards to recover the LMW bioactive compounds. In a recent study by Sørensen et al. (2015), six membrane stages (0.2 µm MF, 50, 20, 10 and 1 kDa UF and NF) were investigated for fractionation of the VC brine. They recovered 42% of the brine (permeate from NF) containing salt and acetic acid ready for reuse as a substitute for fresh water and chemicals. Furthermore, they reported a total waste reduction of 62.5%, while the protein was concentrated 30 times and the amino acids and smaller peptides were concentrated 11 times. Considering this in relation to Paper I where a relatively low protein content was found in VC, yet, with fairly high percentages of the antioxidative amino acids, these six membrane stages might be optimal for recovering the bioactive amino acids from VC. The study by Sørensen et al. (2015) confirmed that membrane technology is a useful method for both wastewater reduction and recovery of protein and amino acids. However, in contrast to our study, the membranes they tested were not ceramic, but different polymeric ones. As discussed in **Section 3.2.7**, ceramic membranes, compared to polymeric ones, have higher thermal, mechanical and chemical resistance, and those made of SiC are extremely stable in harsh conditions, which are an advantage when considering e.g. repeated aggressive cleaning (Zhou et al., 2011). Still, the challenges we were facing, i.e. clogging of the membrane and initial fast decrease in flux, was also described by Sørensen et al. (2015). Recently, several research groups have studied the production and applications of ceramic membranes (Nakamura and Matsumoto, 2013; Facciotti et al., 2014; König et al., 2014) and in the study by König et al. (2014), nearly defect-free SiC membranes for UF were fabricated. This study shows the possible improvements of these membranes which could solve some of the challenges faced in our study. The membranes they fabricated had MWCO between 35-100 kDa, whereas the membranes used in Paper A and II had MWCO estimated to be around 200 kDa with a nominal pore size of 40 nm. It should though be noted, that according to the membrane manufacture, their membranes have been optimised since we ran our tests and it is believed that better separation may be obtained today (LiqTech, 2014). Bearing in mind that the bioactive compounds are usually 2-20 amino acids in length, cf. **Chapter 1**, and the average weight of a peptide is generally considered to be equal to the number of amino acids x 110 Da, the optimal cut-off value would be ~2.2 kDa. With a MWCO >200 kDa we have not

been able to separate the bioactive compounds from the HMW fraction. The lower pore size obtained by König et al. (2014) seems more applicable, and even smaller pore sizes might be produced in the near future, which could further improve the separation of these bioactive compounds.

Membrane separation processes, discussed in **Section 3.2.2**, have also been studied by Taheri et al. (2014), who previously applied this technology for fractionation and recovery of antioxidative protein from herring brine. In this study, one brine, TSa, were analysed after precipitation of the protein (at pH 4.5) and subsequent fractionation using UF with MWCOs of 50, 10, and 1 kDa. They found that the LMW fraction (10-1 kDa) had high radical scavenging activity, and this was significantly ($P < 0.05$) higher than both the >50 and 50-10 kDa fractions. Consequently, this study confirms our result from Paper I; that TSa herring brine is a potential natural source of radical scavengers, as well as showing the need for smaller pore sizes than the ceramic membranes offer today.

In relation to the antioxidant activity found in the study by Taheri et al. (2014), it should be noted that the salt levels were much lower than in the TSa brines tested in Paper I, II, III and B, as they applied a washing step of the retentate which decreased the salt level. Salt is known to have some antioxidant activity, which was clearly shown by the control samples in Paper B, in which salt was better in preventing both lipid and protein oxidation compared to no addition of salt. This indicates that salt is to be considered carefully when investigating antioxidant activity, and hence as described in Paper I, all antioxidant and enzymatic assays used in this PhD-work was tested for the effect of high salt content before conclusions were made.

In Paper III the aim was also to fractionate the herring brine, but in contrast to Taheri et al. (2014), we were analysing both TSa and the two spice containing brines, TSp and SC. Moreover, after dialyse the <10 kDa fraction were further fractionated by SEC into 94 fractions which were all analysed for antioxidant activity and protein/peptides and phenolic content. As reported in Paper III, the three brines had almost identical profiles regarding protein/peptides, total phenolic content (TPC) and the antioxidant activities measured. Yet, the level detected in SC was generally lower than the levels detected in TSa and TSp. For all three brines, the majority of the peptides eluted in three different peaks, P1, P2 and P3, which also contained the majority of the antioxidant activity, especially the radical scavenging activity. Similar results were reported by Falkenberg et al. (2014), in which extracts of salmon were fractionated and analysed for peptides and radical scavenging activity by nearly the same SEC set-up as used in Paper III. They found four peaks that contained all the radical scavenging activity, and these peaks corresponded with the peptide peaks. However, in contrast to Falkenberg et al. (2014), Paper III also provide the TPC levels in all 94 SEC-

fraction, and from these results it is evident that the radical scavenging activity is not just due to peptides, rather this antioxidant activity is linked to the phenolic content.

Further investigation of the pooled fractions (P1, P2 and P3) included amino acids analysis, phenolic acid analysis and identification of the peptide sequences present. P1 had some reducing power and the highest amino acids content (1.2 g/L in SC and <3.5 g/L in TSa and TSp). However, the amino acids present in P1, i.e. Ser, Val, Gly, Asp, Gln or Lys, are not part of the group of amino acids known to be antioxidative, cf. **Section 4.2**. However, peptides containing branched-chain amino acids, e.g. Val, were reported to have antioxidative activity and basic amino acids, e.g. Lys, were reported to be electron acceptors, cf. **Section 4.2**. These two amino acids were present in the identified peptide sequences in P1. Furthermore, whereas a variety of amino acids were detected in all three P1s, only two peptide sequences were identified in TSp and one in SC, which could be caused by a lack of ability of the peptides to “fly” during the MALDI-TOF analysis. TSa-P1 which had the second highest amino acids content did not reveal a peptide sequence. The reason for this is unknown, however, it could be, that all the amino acids detected are present as single amino acids and not as part of peptides, or it could again be the inability of the peptides to “fly”. Further analysis is needed to check if all amino acids are present as free amino acids or if the recovery and up-concentration procedure could be optimised to obtain higher concentrations of peptides which could increase the chance of peptides “flying” during the MALDI-TOF analysis.

The three P2s had high total phenolic content and high radical scavenging activity. Moreover they all contained gallic acid, whilst TSp also contained caffeic, vanillic and ferulic acid. From the amino acids analysis we found that the antioxidative aromatic amino acids Phe and Tyr were present in TSp-P2, and Tyr in TSa-P2, however, no amino acids were detected in SC-P2. In contrast, SC-P2 was the only P2 sample in which a peptide sequence was detected. Resembling P2s, the pooled P3s had notable phenolic content and corresponding radical scavenging activity. They contained 2,5-Dihydroxybenzoic acid and in case of TSp also caffeic and vanillic acid. Thus, as reported by Taheri et al. (2014), we also found high radical scavenging activity in the <10 kDa fraction, and there was a clear correlation between the phenolic content and the radical scavenging activity. In general, the brines are believed to be a complex mixture of several breakdown products of herring protein, and thus a higher number of peptides were expected compared to the low number of peptides identified in the MS/MS analysis. Yet, as discussed above all the peptides present in the nine pooled samples might not have been detected, and the amino acids detected in the total amino acids analysis might not all be stemming from peptides. Therefore more work is needed in this area to obtain knowledge about the LMW peptides in these brines and to evaluate the potential antioxidant activity of the peptides. Nevertheless, the peptides identified in the present study consisted of three to six amino acids, and in SC mainly the tri-peptide HDF was found. Both His and Phe are known antioxidant active amino acids, and this peptide might be responsible for the

radical scavenging activity detected in SC-P3. In comparison, the antioxidant active sequences from herring by-products reported by Pampanin et al. (2012) (see **Table 4.a**), contained mostly Gly and Pro.

The main reason for including both TSa and the spice containing brines in the experimental work related to Paper III, was to analyse whether the spices contributed to the antioxidant activity found in the brines (TSa vs. TSp) or if the major antioxidant activity was a result of peptides present in the brine (TSp vs. SC). From the results obtained in Paper I, it was evident that more protein was present in the barrel-salted brines than in the fillet ripened brines and that in general the antioxidant activity in TSa and TSp were more pronounced than in SC. As discussed in **Section 4.2.2**, spices often contain antioxidative phenolic compounds, and as the amount of spices added to the spice-cured versions is relatively high, these active phenolic compounds could add to the total antioxidative activity. However, this was not observed. From Paper III it seemed that the spice-mix added to the herring brine had some minor influence but the major contributor to the antioxidative activity was indeed the peptides. Nevertheless, some difference between TSa and TSp was detected. Although these two brines had identical protein concentrations in the raw brines and had matured for equally long times with the presence of endogenous proteolytic enzymes, TSp had significantly ($P < 0.05$) more protein in the < 10 kDa fraction compared to that of TSa. One reason might be the addition of sugar in TSp. It has been shown, that the presence of sugar can increase the protease activity in a soil test (Rangaswamy et al., 2009), and thus the sugar could have affected the enzyme activity during ripening and hence promoted the degradation of the protein to smaller peptides below 10 kDa. Still, only the < 10 kDa fraction was studied and this leaves all compounds larger than 10 kDa undetected. For example, there might be polyphenols in this > 10 kDa fraction responsible for antioxidant activity. Indeed, from Table 1 and 2 in Paper III it can be calculated that compared to the total phenolic content (TPC) in the raw brines, the phenolic acids detected by HPLC is only 2.3% in case of SC, 31% in case of TSp, and 44% in case of TSa, respectively. Some of the TPC not detected as phenolic acids might, however, not be simple phenolics, but rather more complex (poly)phenols and therefore not detected. It could also be the presence of aromatic amino acids from protein, which add to the TPC level, but are not phenolic acids.

With this study we gained new knowledge about the LMW compounds in the brines generated during production of both the traditionally barrel-salted herring, with and without spices, and the fillet ripened spice-cured herring. Thus, with an optimised separation method of the LMW compounds these protein/peptide fractions could be fully recovered and used in e.g. functional foods or nutraceuticals. However, an important note is that the separation method should ideally be implemented at the local herring production as part of their process.

In the second supporting paper to this thesis, Paper B, the usability of the brines without any separation was tested. TSa, TSp and SC were tested for their ability to act as a source of natural antioxidants; as coating agents for frozen herring and as additive in fresh herring mince. The brines were tested without pre-treatment, and with pH-adjustment to 2 and 11. The acid treated brines were not applicable due to a very low yield of solubilised protein, and the alkaline treated brines did not lead to any additional antioxidant activity compared to the un-treated brines. The fact that the low pH-adjustment resulted in very low yield of solubilised protein is in line with the reported protein concentration measured in VC in Paper I. Far less protein was detected in VC compared to the brines without acetic acid. This might be a result of protein denaturation caused by the acetic acid, as discussed in **Chapter 2**, but it might also be due to precipitation of the protein in solution by the acid, and thus the protein are no longer soluble and hence not detected. Therefore, acidification of the brines to pH 2 might have denatured and precipitated almost all protein which resulted in low level of solubilised protein.

In this work we showed, that both TSa and TSp (at native pH) are potent coating agents, as they prevented oxidation of the frozen herring better than coating with both SC and water. In contrast, the SC brine was significantly better in preventing oxidation, both lipid and protein, in the fresh herring mince than both TSa and TSp. The reason for this might be found in Table 1 from Paper III. The raw SC had a higher TPC than the two traditional barrel-salted brines; 0.26 mg/mL versus 0.16 mg/mL. Further, the TPC/protein ratio was 8 in the case of SC, whereas only 3.9 and 3.6 for TSp and TSa, respectively. Therefore, these phenolic compounds present in SC could have had ideal conditions (pH, matrix etc.) in the herring mince and thus preventing the oxidation of the mince. In contrast, the glazing treatment might not offer the optimum conditions for the phenolics in SC, for which reason they might not be able to protect the herring to the same extent as in the mince. The two barrel-salted brines both have higher protein content and higher radical scavenging activity and reducing power than SC, which might cause the good oxidative protection of the frozen herring. Still, more detailed studies are needed in order to fully understand the different antioxidant mechanisms in the two set-ups tested here. All in all, the results obtained in Paper B demonstrate that the application of the different process waters from the different end products have diverse antioxidant potential and thus the antioxidant activity should always be tested in the matrix in which its use is intended, as the matrix conditions are important for the antioxidant activity (Arts et al., 2001). Furthermore, these results revealed the opportunity for using the brines in their native form without any separation step.

In summary, the work done in this thesis add to the knowledge of the usability of the herring brines, especially the traditionally barrel-salted products, TSa and TSp, and the fillet ripened spice-cured product, SC. However, in a recent review by Olsen et al. (2014) the use of by-products as new resources was discussed and it was stated that today only a few high-value products are on the market. The authors point at an overestimation of the market, too small

amounts of high quality by-products available on a regular basis, and high cost related to purifying biomolecules of interest. Nonetheless, before they even reach the industrial waste streams, marinated herring brines are food grade and follow hygienic standards. Consequently, with the new knowledge obtained through this PhD work, continued efforts to obtain an online separation method, and subsequent recovery step for the bioactive compounds, the usage of valuable compounds in the herring brines should be accomplished.

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7. Conclusions and perspectives

The main goal of this thesis was to characterise the herring brines generated during the last ripening step in the production of marinated herring, and to apply two separation technologies which have not previously been used for these types of wastewaters. Furthermore, the aim was to identify potential bioactive compounds in the herring brine suitable for use in the food or nutraceutical industry.

The major conclusions obtained in each of the five papers related to this thesis are presented in **Figure 7.a**, and combining these conclusions enables the overall working hypothesis, presented in **Chapter 1**, to be answered;

Yes, the brines generated during the ripening of marinated herring, especially the traditionally barrel-salted versions, TSa and TSp, and the fillet ripened spice-cured version, SC, contain bioactive compounds, such as protein/peptides, amino acids and antioxidant and enzymatic activity. Moreover, they are a source of nitrogen, phosphor and iron as well as phenolic compounds.

Yes, the bioactive compounds could be recovered using the ceramic UF membrane, but further optimisation is needed to obtain an optimum separation.

No, the electro-flocculation treatment is not suitable as a treatment technology when recovery of bioactive compounds is of interest, as this technique results in high aluminium concentration in the treated brine.

Yes, the bioactive compounds in the herring brines could be used as an added value instead of being discarded as waste. The TSp and TSa were good coating agents whereas SC can be used to prevent oxidation in fresh herring mince.

All in all, the brines generated during the final ripening step in the production of marinated herring are a source of protein, peptides and amino acids with antioxidant activity, which can be used e.g. un-treated as coating agents or as an additive in fresh herring mince. The pre-selected separation technologies tested should be further optimised in the case of ceramic UF to avoid clogging and to increase the flux. In case of the EF, this technology is fast and efficient in treating the wastewater, however, not if the main purpose is recovery.

Currently, up to 119 kg protein and 44 kg fatty acids are discarded, from boat to glass jar, as wastewater per ton herring processed. With the new knowledge obtained in this work, the starting point for creating value-added products from herring brines and by that increasing the revenue for the herring producers has been defined.

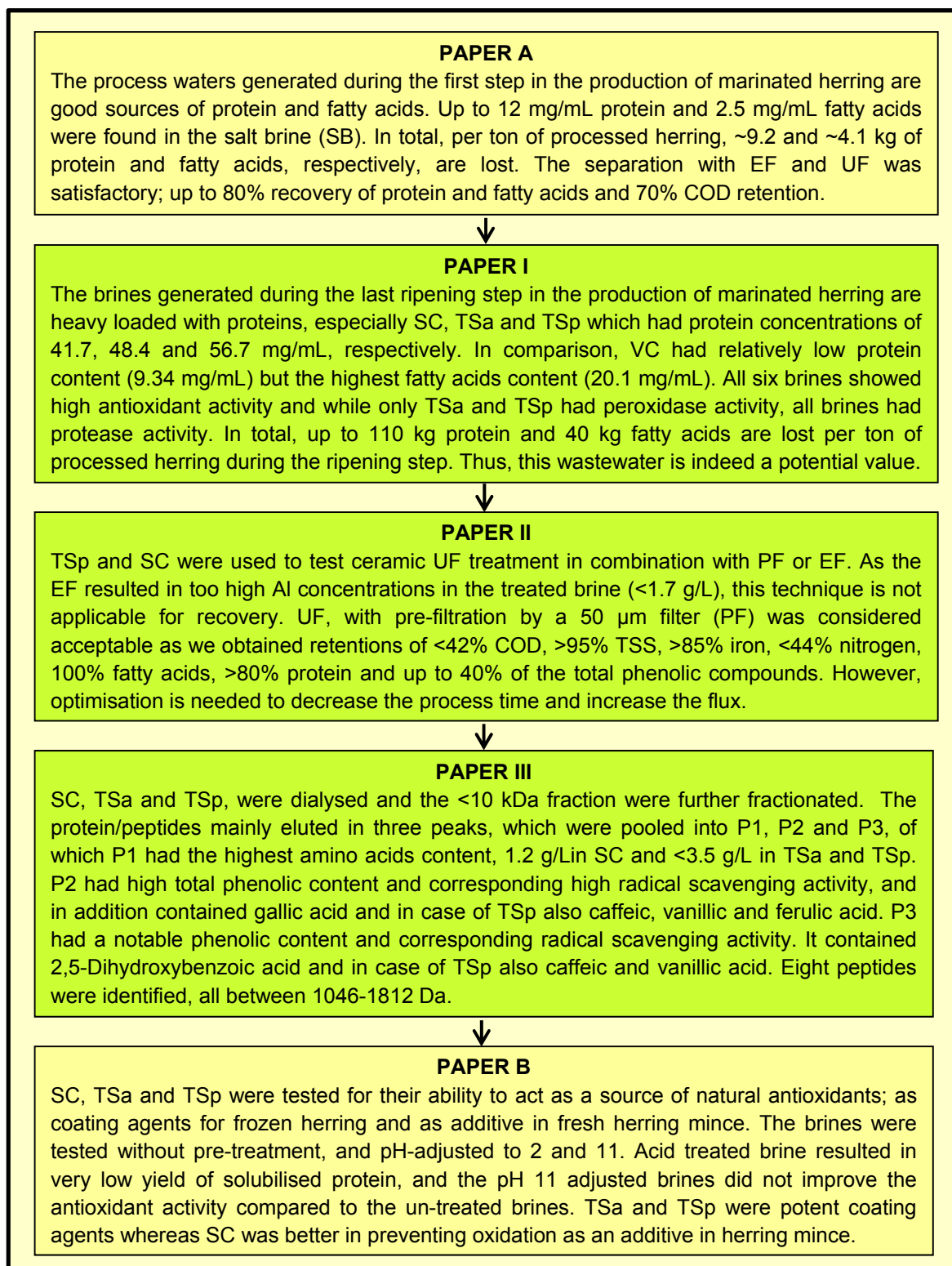


Figure 7.a. Major conclusions obtained from the experimental work conducted in the thesis.

As future perspectives, some studies are recommended based on the results presented in this thesis. The main goal would be to gather knowledge of where and how to utilise the herring brines previously considered as waste;

In relation to separation and recovery

- Study the recovery of antioxidant amino acids from the VC brine, e.g. with a tricanter centrifuge followed by ceramic UF
- Test an iron electrode in the EF unit, and analyse the treated brine for iron-induced oxidation
- Test a tricanter for the separation of the oils, LMW protein fraction, and HMW protein fraction from all four brines; VC, SC, TSa, TSp
- Re-run the separation test with the improved ceramic UF membranes

In relation to bioactive compounds in the brine

- Analyse the phenolic content in fresh, unused spice brine, to compare the antioxidant activity of this brine with that found in the used brine, and by that identify which compounds are leaching from the herring and which were present already in the fresh spice brine
- Further study of the two desalting brines, D-TSa and D-TSp, to reveal the potential in these brines
- Optimise the preparation for MALDI-TOF analysis to increase the possibility for the peptides to fly, and thus be detected
- Analyse different seasonal batches of herring brine to detect potential differences in the biomolecules herein

In relation to usage of the brine

- Test the use of the brines as medium for microorganisms
- Study the addition of the LMW antioxidative fractions as additives to different food matrixes, e.g. yoghurt, fitness bars etc., and test the sensory aspect of this addition
- Study the HMW fraction more in detail in relation to potential use of the high protein content as feed for e.g. chickens
- Purify the n-3 LC-PUFA for application in food and fish-oil tables
- Test the production of fermented fish sauce made from the herring brines

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